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THE DISTRIBUTION OF SOME ELECTROLYTES IN THE HEART

Studies on normal and vagus-stimulated hearts

BY

BO G. DANIELSON

UPPSALA 1964

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Translated

by

Maud Marsden

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PART I

CHAPTER I

Introduction

Already at the beginning of this century the electrolyte content and electrolyte distribution in biological tissues began to arouse attention. OVERTON (1902, 1904) and HÖBER (1906) studied the mechanism which hindered the transport of sodium chloride from the tissue spaces into the cells and the efflux of potassium phosphate from the muscle fibres. They advanced two theories, one of which postulated that the fibres were surrounded by a semipermeable membrane which resisted the passage of sugar and certain salts (more permeable for potassium salts than for others). According to the other theory muscle tissue was regarded as a colloidal solution of gelatin and fibrin, lacking any enveloping membrane (for a modern similar theory, see LING, 1962).

FENN *et al.* (1934) considered that all chloride in frog striated muscle was extracellular, and calculated from the chloride contents of the muscles and plasma the *chloride space* in the muscle. (The chloride space in a tissue = the ratio of the chloride content in the tissue to the chloride concentration in the plasma). It was assumed that the interstitial or extracellular fluid had the same concentration as plasma, the chloride space was thus a measure of the extracellular space. The distribution of other electrolytes between the cells and the extracellular space was calculated from the chloride space and their concentration in the tissue and plasma.

During the last 25 years analytical methods have improved considerably (by the introduction of radioactive isotopes, flame photometry, spectrophotometry etc.). The notion that the cell is impermeable to ions has been superseded by the concept of a continual exchange of matter between the cell and its surroundings. It is considered that the concentration diffe-

rences are maintained not only by passive physico-chemical processes but also by metabolic reactions involving enzyme activity.

It is known that sodium and chloride ions are found mainly in the interstitial or extracellular fluid, and that potassium and phosphate are intracellular. In recent years special attention has been paid to the problem of how this asymmetry of ionic distribution between the intra- and extracellular phases arises and can be maintained (see further the introduction to part II).

A tissue may be divided into two morphologically different phases, namely an extracellular and an intracellular phase. The *intracellular* phase is considered to consist only of those cells specially concerned with the function of the tissue (e. g. for the muscle, muscle cells). Assigned to the *extracellular phase* are the supporting connective tissue structures which surround the cells, and certain fluids. Certain cells are also usually included in the extracellular phase, namely the fibroblasts in the connective tissue, and the cells of the capillary walls.

The extracellular phase thus consists of two parts, the solid constituents (mainly proteins in connective tissue, capillary walls and nerve fibres) and fluids (*interstitial fluid*, blood, lymph and the fluids in the connective tissue cells). The *interstitial fluid*, which constitutes the *greatest part* of the extracellular volume, consists of water derived from the plasma and contains ions and small molecules which also move outwards from the plasma, i. e. the fluid resembles an ultrafiltrate.

The extracellular volume (ECV) can be calculated if the total quantity and the extracellular concentration of a substance, which is distributed in the entire extracellular space without entering or becoming attached to the cells, is known. No substance ideal for this purpose has as yet been found. It is usual instead to determine approximate extracellular spaces by means of substances which are *mainly* distributed in the physiological extracellular space and to define the spaces found according to the substance used, e. g. the sodium space, mannitol space etc.

Two groups of substances have been used in attempts at

determining the extracellular space: 1. *Electrolytes*, whose distribution resembles that of sodium or chloride (thiocyanate, iodide, bromide, sulphate and thiosulphate ions). 2. Small *non-electrolytes*, which pass through capillary walls but do not, or only to a very small extent, penetrate the cells (mannitol, see PAGE, 1962; sucrose, see ROBERTSON and PEYSER, 1956; raffinose and inulin, see BOZLER, 1959, 1961).

Substances belonging to the first group are also distributed, even if to only a small extent, intracellularly, and the ECV determined with these substances is therefore somewhat too large. The ion most frequently used, thiocyanate, has the disadvantage that it binds to the plasma albumin (SCHEINBERG and KOWALSKI, 1950). Thiosulphate has also been suggested, since it does not bind to proteins (KOWALSKI and RUTSTEIN, 1952), and radioactive sulphate has also been used (WALSER, SELDIN and GROLLMAN, 1953, 1954).

As a rule the turnover of ions in the physiological extracellular space is rapid (for the sulphate and sodium ions the diffusion coefficient is about 60 % and 80 % of the diffusion coefficient in free solution, respectively, see JOHNSON, 1955). Some of the non-electrolytes, belonging to group 2, are excreted rapidly, and others are metabolised so that they also disappear from the plasma. The distribution volume of inulin, for example, is often smaller than the corresponding chloride space.

The majority of the studies on ion distribution have been made on striated muscle but several studies on heart tissue from different animals have also been made. Heart tissue in mammals contains more water, sodium and chloride and less potassium than the skeletal musculature, which may indicate a larger extracellular space (MANERY, 1954).

Since the literature on the electrolyte distribution in different tissues is very extensive, reference may be made to review articles for more complete reference lists (MANERY, 1954; BERGSTRÖM, 1962, among others).

Relatively few studies have been devoted to comparisons of the electrolyte composition in different regions of the heart. The aim of the present investigation was therefore to *attempt to elucidate the electrolyte distribution in the heart and to*

compare it with the distribution in skeletal muscle. A further aim was to *attempt to determine the physiological extracellular volume and on the basis of the values obtained to calculate the intra- and extracellular electrolyte concentrations.*

Knowledge of these conditions appears to be necessary for the understanding of the nature of the spontaneous rhythmicity of the heart, as this seems to depend on or at least linked to the ionic distribution and fluxes (see TEORELL, 1962). It also seems important to study the electrolyte distribution in the heart in other states where the rhythmicity is altered. The heart rate can be easily changed by vagal stimulation. Studies were also therefore made *of the electrolyte distribution in the vagal stimulated heart and the exchange of ions between the heart and the blood on vagal stimulation in an attempt to find a basis for chronotropic changes* (see further the introduction to part II).

In these experiments the heart and skeletal muscle of toads were studied. These animals have the advantage that the primary pacemaker is localized to a limited anatomical structure. viz. the sinus venosus, and also that the atrium and ventricle are relatively homogeneous tissues.

CHAPTER II

Methods

A. GENERAL METHODS

a) *Experimental animals*

Toads (*Bufo bufo* or in one series *Bufo viridis*) were used for the experiments. The animals were caught during May–August and were kept at 8–14°C. They were given no solid food but had access to water. The experiments were started in May and continued until February of the following year. Studies were made of different regions of the heart (sinus venosus, atrium and ventricle) and the skeletal muscle (m. sartorius) was also studied.

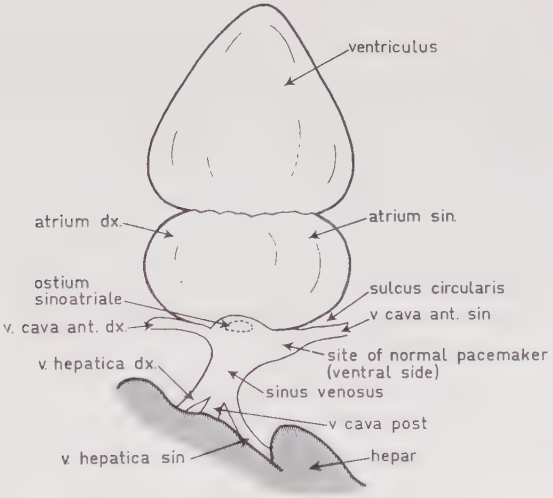

Figure 1. Toad heart (*Bufo bufo*), semi-schematic. The atria and ventricle have been displaced cranially in order to show the sinus venosus.

b) *The toad heart*

Fig. 1 shows a semi-schematic diagram of the toad heart, which consists of the sinus venosus, the atrium divided into two chambers, and the ventricle. For further anatomical details reference may be made to ECKER-WIEDERSHEIM (1904).

1. *Structure of the toad heart*

For a microscopic description of the anatomy of the toad heart, see KISCH (1961). The *sinus venosus*, which is the normal site of origin of the electrical impulses in the toad heart, is characterized by a layer of striated muscle with the same histological properties as the musculature in the other regions of the heart. Between the single endothelial cell layer, which is considered to correspond to vascular endothelium (LINDNER, 1957), and the muscle fibres, as also between the individual muscle fibres, are large quantities of longitudinal and transverse collagen fibres. The *atrium* has to a great extent the same construction as the sinus, but the muscle layer is considerably thicker and the connective tissue content smaller. The *muscle of the ventricle* has the same histological structure as the atrium and sinus.

There is no *specific conducting system* in the toad, as also in the other lower vertebrates, with the possible exception of certain turtle species (FÄNGE, 1963). KISCH (1961), however, found profuse quantities of non-myelinated nerves in all regions of the heart.

KISCH (1961) never observed either *capillaries* or any white or red blood cells between the muscle fibres in any part of the heart, and this was considered to indicate that the toad heart lacks a coronary system (ECKER-WIEDERSHEIM, 1904).

The extracellular space begins in the sub-endocardial region and is in communication with the spaces between the muscle fibres (LINDNER, 1957).

Morphologically the borderline between the intracellular and the extracellular phase is difficult to define. In spite of the fact that the cell boundary is difficult to determine exactly, HUTTER and TRAUTWEIN (1956) stated that the heart muscle cells in the frog had a diameter of about 5 μ, and IWAMOTO

(1959) gave the same value for the fibres in the sinus venosus of the toad.

Simpson and Oertelis (1962), in electron microscopic studies on the ventricular musculature of the sheep heart, found that the sarcolemma had invaginations, which seemed to connect the intra- and extracellular spaces. Spiro (1962) made also similar observations.

An account of the normal morphology of *skeletal muscle* may be found in Maximow & Bloom (1942).

2. *The physiology of the toad heart*

Nutrition

Since the toad heart lacks a coronary system (Ecker-Wiedersheim, 1904) the heart tissue receives its nutriment by means of the direct transport of solutes from the plasma in the heart lumen through the sponge-like structure in the wall. The sinus wall is only about 100 μ thick, and the nutritional conditions are presumably quite adequate. The wall thickness of the other heart regions is considerably greater.

The localization of the pacemaker

Iwamoto (1959) found that the primary pacemaker was localized to the ventral part of the orifice of the left anterior vena cava in the sinus venosus (see Fig. 1). Hutter and Trautwein (1956) maintained that the pacemaker was normally situated in the left region of the sinus venosus.

c) *Dissection of the toads*

The toads were pithed, after which the heart was dissected free from connective tissue and pericardium. The sinus venosus, atrium and ventricle were removed, as also were skeletal muscle samples (sartorius muscle). The sinus venosus and also double samples of atrium, ventricle and skeletal muscle were lightly blotted on filter paper and then weighed (see below). (Approximate weights of the preparations: Sinus 5–15 mg, atrium 15–35 mg, ventricle 35–75 mg, skeletal muscle 40–150 mg of wet tissue.)

d) *The collection of blood samples and the preparation of plasma samples*

Before the heart was dissected free, blood samples were collected from the left aorta into heparinised capillary tubes, the ends of which were then sealed in a flame and centrifuged at ≈ 9600 g for 3 mins. The tubes were then cut open and the plasma samples were removed with calibrated constriction pipettes.

e) *Weighing*

The tissue samples were freed from blood and tissue fluid by blotting on filter paper. Duplicate samples of ventricle and skeletal muscle were enclosed in polyethylene tubes (weight of tube about 1.2 g), after which they were weighed on a balance accurate to 0.1 mg. The plastic tubes prevented evaporation. The sinus and atrium preparations were weighed repeatedly on an electro-balance (Cahn Instrument Co., Minnesota Ave., Paramount, Cal., U. S. A.; max. range 40 mg, accuracy 30 μg, precision 8 μg) after periods of different lengths, after which the initial weight was determined by extrapolation. In this way the error caused by the evaporation could be reduced. The error was proportionally greatest for the sinus venosus preparation. The weight curve is shown in Fig. 2, where it may be seen that the evaporation is approximately proportional to time (at least the first six minutes).

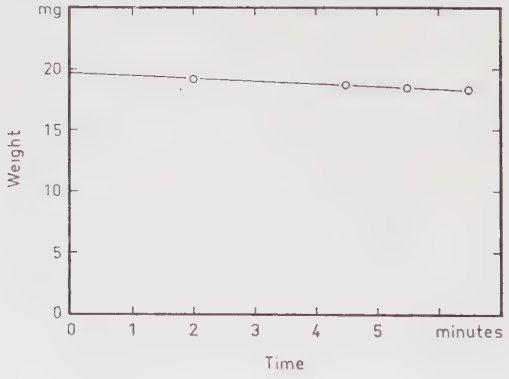

Figure 2. Weight curve of a tissue sample extrapolated to zero time.

The evaporation loss, which is presumably exponential, amounts during the first 6 minutes to about 6–7 % of the initial wet weight.

f) *Reference units for the concentrations of test solutes*

The amounts of the different test substances are given as concentration per unit wet weight of tissue. It is also possible to use fat-free dry substance (DARROW, HARRISON and TAFFEL, 1939) or non-collagenous nitrogen as the reference units (LILIENTHAL *et al.*, 1950).

Since data on the neutral fat content was not found in the literature, this was investigated (for method see page 20, and results Table 1, page 21).

Since it was found that the water content varied very little between different toads (see chapter 7, table 14), and the neutral fat contributed to only a very small part of the wet weight (see Table 1, page 21), the wet weight seemed a valid reference unit.

B. CHEMICAL ANALYSES

a) *Determination of sodium and potassium*

Sodium and potassium were determined by flame photometry. Heart and muscle tissue were first ashed by boiling in strong acid, while plasma samples were merely diluted with water.

1. *Solutions*

Acid mixture. This consisted of 6 parts concentrated HNO_3, 4 parts concentrated $HClO_4$ and 1 part concentrated H_2SO_4 (all acids quartz distilled).

To prevent bumping quartz fragments were used. These had been boiled with sulphuric acid for two hours, washed with H_2O, boiled in HNO_3 for 30 mins and dried at 100°C.

Standard solutions of sodium and potassium were prepared by drying analysis-grade sodium and potassium chlorides at 600°C for about 2 hours, after which the substances were cooled in a desiccator. The samples were weighed and dissolved in double-distilled water.

2. *Burning of tissue samples*

Sinus venosus preparations from two toads were combined. The atria from one toad consisted one sample. The ventricle and skeletal muscle samples were divided into two. Two modes of procedure were used in the burning process. In one of the methods the atrium and sinus preparations were placed in quartz crucibles (in order to obtain a more favourable ratio of sample/blank) and the ventricle and muscle preparations in pyrex glass beakers, 3 ml of acid mixture (the same amount for all samples) and two quartz fragments were added, and the vessels, covered with watch glasses, were then heated electrically in a draught cupboard. After the burning the residue was dissolved in double-distilled water (for sinus 5 ml, for atrial preparations 10 ml and for ventricular and skeletal muscle samples 25 ml).

In the other burning method the tissue samples were placed in 50 ml Kjeldahl flasks, 3 ml acid mixture and two quartz fragments were added, and the flasks were then placed in a suction apparatus as described by NYDAHL (1949, 1950) (see Fig. 3), to safeguard against explosive combination of the perchloric acid vapour and organic material in the environment. After burning, the residue was dissolved as previously.

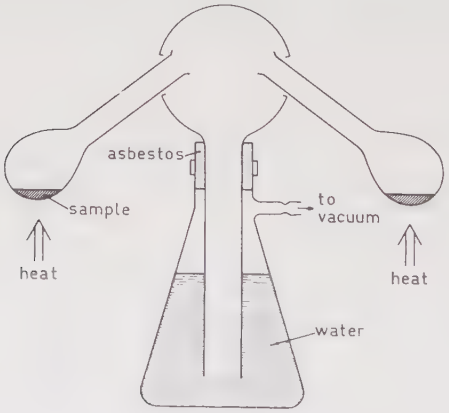

Figure 3. Apparatus for digestion with acid mixture as according to NYDAHL (1949, 1950). The seal between the suction flask and the neck of the upturned round flask consists of wet asbestos.

In both procedures blank samples were prepared in the same way as the tissue samples except for the presence of the tissue.

Flame photometric analysis was performed as described below.

3. *Preparation of plasma samples*

Duplicate samples of 25 μl plasma were measured with calibrated constriction pipettes and diluted with 5 ml double-distilled water, after which flame photometric determination was made as described below. No surface active agent was added, and neither were the plasma proteins precipitated prior to the analysis of sodium and potassium. BERGSTRÖM and HULTMAN (1962) showed that the sodium content in human plasma before precipitation was about 4.5 % lower than after precipitation of plasma proteins. In the toad, where the plasma protein content is very low (see chapter IV), this interference can be regarded as negligible.

4. *Flame photometric determinations*

The concentrations of sodium and potassium were determined with a flame photometer (flame photometer, Eppendorf, Netheler & Hinz, GmbH, Hamburg)—propane/air flame. With this gas mixture no interferences between the sodium and potassium determinations were obtained. Double-distilled water was used as the zero reference.

5. *Error of the method*

Six samples consisting of 0.2 ml human whole blood were burned, after which the sodium and potassium contents were determined. The coefficient of variation was $\pm$ 1.5 % for the sodium analyses and $\pm$ 2 % for the potassium analyses.

b) *Determination of chloride*

The chloride content in the tissue and plasma samples was determined after burning. The preparations were charred after the addition of an excess of calcium acetate so that a basic

ash should be obtained (thus preventing the release of hydrogen chloride during the heating process). Most of the resulting ash was dissolved in a water-alcohol mixture. The chloride content was then determined by mercurimetric titration.

1. *Solutions*

Ethanol, absolute 99.5 % (chloride-free).
s-diphenylcarbazone (Eastman Kodak 4459), 0.1 % solution in absolute ethanol.
Brom cresol green (Merck AG, Darmstadt, Germany) 0.1 % in H_2O (stock solution).
Nitric acid 0.05-M.
Mercuric nitrate 0.01-M.
Sodium chloride 0.02-M.
These solutions were prepared as according to Danielson (1963).
Calcium acetate solution 0.25-M.
The calcium acetate solution was prepared by placing 50 g $CaCO_3$ according to Lawrence Smith in a 2 litre measuring flask and dredging the substance in about 800 ml distilled water, after which 60 g distilled glacial acetid acid (approximately 65 ml) was added. The calcium carbonate was dissolved by boiling, and the solution was then cooled, and 1 litre 95 % ethyl alcohol was added. The solution was then made up to 2 litres with distilled water.

In the preparation of the solutions double quartz distilled water was used in all cases; solutes were analytical grade except when stated.

2. *Preparation of samples*

The heart and muscle preparations were placed in small quartz crucibles (diameter 14 mm, height 25 mm), and 0.3–0.5 ml 0.25-M $Ca(Ac)_2$ solution was added. The samples were dried under a heat lamp to prevent boiling during the charring process, which was done in an oven for 12–15 hours at 560°C. The ash was dissolved in a mixture of water and alcohol as described below:

1 ml distilled water was added to the sample in the quartz crucible and the ash was stirred with a glass rod, after which the solution and deposit were poured into a centrifuge tube. The remaining ash was stirred further in 1 ml distilled water and poured into the centrifuge tube. In order to transfer the deposit quantitatively into the centrifuge tube the crucible was washed out with absolute alcohol (5–6 ml).

The sample volume was adjusted to 12 ml with alcohol and the sample was then centrifuged. The supernatant solution was poured into a 25 ml beaker. The deposit was washed once with a water-alcohol mixture (1 ml H_2O and 6 ml alcohol) and the washing fluid was added to the remaining sample.

The plasma samples were prepared in the same way as the others. 50 μl were burned with 0.3–0.5 ml calcium acetate solution to obviate the influence of the proteins on the final determination.

3. *Titration of chloride*

Chloride was determined by means of mercurimetric titration in a mixture of ethanol and water as solvent at a pH of 3.5 with photometric estimation of the end point (Danielsson, 1963).

4. *Error of the method*

10 samples (weight of each sample about 75 mg) from the same rabbit muscle, cut into small pieces, were burned with calcium acetate solution, after which the samples were treated as above. The coefficient of variation was $\pm$ 5 %. One reason for the large variation coefficient may be that the actual chloride content may vary within the same muscle (cf. chapter IV, page 37). In a series of 5 identical plasma samples of 50 μl each the coefficient of variation was $\pm$ 3 %.

c) *Determination of proteins in the plasma*

The plasma proteins were determined by the biuret reaction as according to Knights, MacDonald and Ploompuu (1962). The biuret reagent gives a blue colour with proteins. The

colour of the sample solution was compared with that of a protein solution with a known content, which was treated in the same way.

1. *Solutions*

The chemicals used were of analytical grade.

Concentrated carbonate-free sodium hydroxide was prepared as according to KOLTHOFF and SANDELL (1961).

Sodium hydroxide, 10 %, was prepared from the above solution.

Biuret solution: 1.5 g copper sulphate ($CuSO_4$, 5 H_2O) and 6.0 g potassium-sodium-tartrate were dissolved in 500 ml distilled water in a measuring flask. 300 ml 10 % sodium hydroxide were added during stirring, and the solution was then diluted to 1000 ml with distilled water.

Albumin solution, 4 %. This solution, used as a standard solution, was obtained by diluting 20 % albumin solution (Kabi, Stockholm, Sweden) with distilled water.

2. *Procedure*

To three tubes containing 50 μl plasma, 50 μl distilled water and 50 μl of a standard human albumin solution (4 %) respectively, were added 2.0 ml biuret solution. The tubes were then shaken and allowed to stand for 30 minutes, after which the absorbance at 540 nm was measured with distilled water as blank. The protein concentration in the sample was calculated from a calibration curve.

3. *Error of the method*

The plasma protein concentration in five samples containing 50 μl of the same plasma was determined as described above. The coefficient of variation was $\pm$ 1 %.

d) *Determination of water and neutral fat contents*

The tissue preparations were dissected out, blotted "dry" and weighed repeatedly, and then dried on small pieces of aluminium foil in an oven at about 90–100°C for at least

2 hours, often 10–12 hours, then re-weighed. The water content was obtained by subtraction of the dry weight from the wet weight at zero time. BERGSTRÖM (1962) reported that drying for 2 hours was sufficient to obtain a constant weight.

Both the water and neutral fat content were determined in certain preparations. The preparations were fixed on to small wire hooks (weight about 10 mg), dried at 90°C for about 10 hours and re-weighed. They were then placed in 10 ml petroleum ether (Baker Analyzed Reagent: boiling point 30–60°C) for 2–3 hours, dried at 90°C for about 30 mins and weighed again. The fat content was determined as the difference between the dry weight and the weight after extraction with petroleum ether. The extraction time should have been sufficient as FLEAR and FLORENCE (1961) found complete extraction of fat after 10 mins in samples weighing 9–16 mg (wet tissue). For the results of the neutral fat determinations, see Table 1.

Table 1.

The neutral fat content of the heart and skeletal muscle. The table shows the mean values ± one standard deviation obtained from the analysis of preparations from 10 toads (only 7 determinations on the sinus venosus).

Preparation	g neutral fat/100 g wet tissue
Sinus venosus	0.7 ± 0.6
Atrium	2.0 ± 1.4
Ventricle	1.1 ± 0.6
Sartorius muscle	0.5 ± 0.3

C. RADIOACTIVE ANALYSES

a) *Gamma spectrometry (analysis of Na^{22}, Na^{24}, K^{42}, Br^{82} and I^{131})*

1. *Apparatus and preparation of samples*

The gamma spectrometer had a $1^3/_4''$ NaI(Tl) well type crystal combined with a photomultiplier (type RCA 6655), the amplification of which did not change with the impulse frequency (ÖBRINK and ULFENDAHL, 1959). The high voltage of the photomultiplier was obtained from aggregates with very good stability (Tracerlab Precision High Voltage Supply, long period stability 0.02 %/24 hours). The supply voltage of the apparatus was also stabilized (228 V ± 0.2 %). The linear

amplifier (Tracerlab RLA-1) had good "non over-load" properties. Two pulse height analysers (Tracerlab RLA-5 S) were used.

The gamma energy spectra were recorded as according to Öbrink and Ulfendahl (1959).

Two types of analysis tubes of polythene plastic were used. The larger type (16 × 59 mm, volume 6.5 ml) fitted exactly into the well of the crystal. (Used in the analysis of Na^{22}, K^{42}, Br^{82} singly and in the analysis of mixtures of Na^{24} and Br^{82} and of Br^{82} and I^{131}.) The samples were inserted directly into the tubes in such a way that comparable conditions of analysis were present for all samples.

The samples were placed in small plastic tubes (outer measurement 8 × 38 mm, volume 1 ml). The sample volume never exceeded that which gives 98–100 % relative counting activity. In the analysis of the Na^{24} and K^{42} mixture the beta radiation was screened off, especially that of K^{42}. Each plastic tube was placed in a brass cylinder (2000 mg/cm^2, which gives small Bremsstrahlung because of low atomic number, 28 + 29) in order to obtain absorption of all beta particles with energies below 4.0 MeV. The brass cylinder containing the sample tube was placed in a larger plastic tube.

2. *Error of measurement*

A Cs^{137} sample was counted 50 times (energy channel = 0.004 MeV set on the steep part of the 0.66 MeV peak), and a coefficient of variation of ± 0.25 % was obtained, i. e. the theoretical counting error (Danielson and Sjöstrand, 1964). This showed the good stability of the apparatus.

In all cases the necessary corrections for radioactive decay were made.

3. *Determination of individual isotopes (Na^{22}, K^{42} and Br^{82})*

In the analyses of individual radioactive isotopes broad energy channels (wide open windows) were used, and it may be assumed that the error in the analysis of one isotope was approximately $\pm \sqrt{n}$, where n is the number of impulses recorded (see Schmeiser, 1961). In the analysis of Na^{22} or Br^{82}

all impulses below 1.50 MeV were recorded and in the analysis of K^{42} or Na^{24} all impulses below 3.5 MeV. As a rule at least 10,000 impulses from each sample were counted.

4. *Determination of radioactive isotopes in mixtures*

The determination of isotopes in mixtures (Br^{82} and I^{131}, Na^{24} and Br^{82} or Na^{24} and K^{42}) was carried out according to the method of Öbrink and Ulfendahl (1959). In order to reduce the error broad energy channels were used, thus giving a higher counting rate. By selecting suitable energy values for the channel boundaries the effects of the instability of the apparatus can be reduced (cf. Danielson and Sjöstrand, 1964). In the analysis of Br^{82} and I^{131} the channel boundaries were chosen such that in channel I all energies below 1.50 MeV were recorded, and in channel II energies between 0.40 and 1.50 MeV. In the analysis of Na^{24} and Br^{82} all energies below 4.0 MeV were recorded in channel I, and energies between 1.0 and 4.0 MeV in channel II. In the analysis of a mixture of Na^{24} and K^{42} energies between 1.1 and 2.1 MeV were recorded in channel I, and energies between 1.1 and 3.5 MeV in channel II. By frequent counting of the standard samples (approximately every fifth sample was a standard), any drift in the channels could be corrected for.

At least 5000 impulses from each isotope were recorded in all samples. The statistical counting error was never greater than $\pm$ 1.4 %, and the total error in the determination of isotopes in mixture probably did not exceed $\pm$ 3 % in any analysis.

b) *Liquid scintillation counting (analysis of C^{14} and S^{35})*

For the analysis of C^{14} and S^{35} a method described by Kalberer and Rutschmann (1961) was used, based on the oxygen combustion method reintroduced by Schöniger (1955).

Preparations containing C^{14} and S^{35} are burned in pure oxygen. The products (carbon dioxide, sulphur dioxide and sulphur trioxide) are then absorbed in a liquid mixture, which is later mixed with toluene containing phosphors for liquid scintillation counting.

1. *Solutions*

The *absorption solution* in the C^{14} and S^{35} determinations was 15 ml of a mixture of 120 ml distilled 2-amino-ethanol, diluted to 1000 ml with analytical grade methanol. The volume used can quantitatively absorb the combustion products from 200 mg substance (including the filter paper). The *scintillator solution* consisted of toluene (Baker's toluene, Analyzed Reagent), to which was added 4.0 g PPO (2.5-diphenyl-oxazol) and 100 mg POPOP (1.4-di [2(5-phenyloxazolyl)]-benzene) to 1000 ml toluol.

2. *Preparation of samples*

For combustion 1 litre pyrex round flasks with tightly fitting glass stoppers (NS 29 or 24) were used. The appearance of the flask and stopper is shown in Fig. 4. A platinum wire was

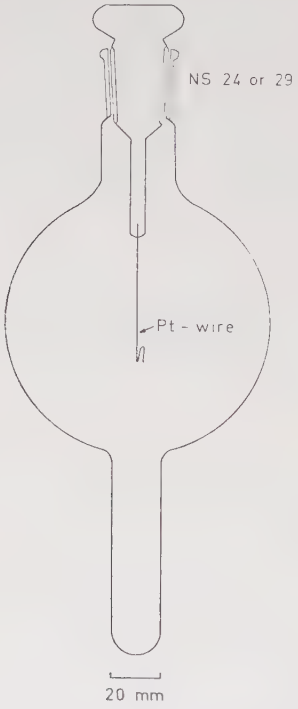

Figure 4. 1 litre burning flask with glass stopper into which a Pt wire is fused.

fused on to the inner side of the stopper. A blind glass tube (length 90 mm, inner diameter 20 mm) was fused on to the bottom of the flask, to contain the absorption solution during the burning process. (One-litre flasks suitable for burning preparations up to 0–250 mg in weight.) Optimal combustion conditions are obtained if only about a third of the oxygen content of the flask is used. The preparations were placed on special burning papers (see Fig. 5) i. e. quantitative filter papers (No. 589 Schleicher and Schuell, AG, Feldmeilen, Zürich, Switzerland) weighing about 130 mg each. (The ash content in the filter paper was approximately 0.01 %.) A piece of platinum net (1.5 × 1.5 cm, 48 mesh, perforated Pt-plate) was placed around the preparation, after which the preparation was fixed on to the platinum wire in such a way that the net was open at the top and sides.

3. *Procedure*

Combustion was carried out as follows:
The flask with the absorption solution was lowered into a thermos vessel with a mixture of dry ice and acetone for 3 minutes, which sufficed to cool the absorption solution to -60°C. Oxygen was then blown into the flask for 1 minute. The contact surface of the stopper with the preparation to be burned was damped with water to obtain an effective gas-tight

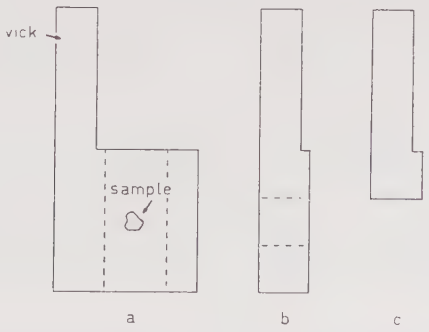

Figure 5. Combustion paper with sample (a) is folded along the dotted lines (a and b) to form a small packet, which is enclosed in a piece of Pt net and attached to the Pt wire on the glass stopper (see Fig. 4).

lock, a corner of the filter paper was lit, and the stopper was lowered quickly into the oxygen-filled flask. After burning, the flask was left in the cooling mixture for two minutes to allow the gas and platinum wire to cool, and the flask was then rotated mechanically for 20 minutes.

10 ml scintillator solution were pipetted into the counting flasks, to which 10 ml of the absorption solution were added, and the samples were then analysed.

4. *Apparatus*

A Packard Tri-Carb Liquid Scintillation Spectrometer, model 314 EX-2 (Packard Instrument Company, LaGrange, Ill., U. S. A.) was used at + 3°C. The voltage of the photomultiplier was 1150.

The stability of the spectrometer was tested as follows: One S^{35} sample was counted 62 times during a period of about 10 hours. The coefficient of variation was ± 0.78 %. The theoretical statistical counting error for the number of impulses counted was ± 0.67 %; i. e. the stability of the spectrometer was good.

5. *Measuring conditions*

The channel setting is shown in Fig. 6. The background was of the order of size of 75 cpm in the large energy channel. The samples were as a rule counted 3 × 10 minutes each, at least 10,000 impulses usually being recorded in the wider channel each time.

The spectral curve for S^{35} was determined. The exact energy values were not determined, only the appearance of the spectral curves being studied. (The abscissal values in Fig. 6 are therefore only approximate.) Since S^{35} and C^{14} have almost the same energies (0.167 and 0.155 MeV respectively), only the spectral curve for S^{35} was determined.

The quotient between the net counts on the two energy channels gives an idea of any variations in quenching from sample to sample, i. e. quenching causes a shift of the whole

spectral curve towards lower energies and some extinction of impulses. The coefficient of variation for the quotient between the two energy channels was ± 1.5 %. The reason for these variations in the quotient may be: 1. Quenching variations. 2. Instability in the electronic apparatus (see page 26).

Lack of differences in quenching is probably due to the large volume of quenching substances such as methanol and ethanolamine, which mask the effects of small variations in the sample quenchers, e. g. water and ash particles. The main reason for the small variations in the quotient between the two energy channels was probably instability of the electronic apparatus.

6. *Error of the method*

To determine the error of the method 10 μl S^{35} solution were pipetted on to five combustion papers, dried, burned and analysed. The coefficient of variation was ± 2 %. (The statistical counting error for the same number of impulses was = 0.5 %.)

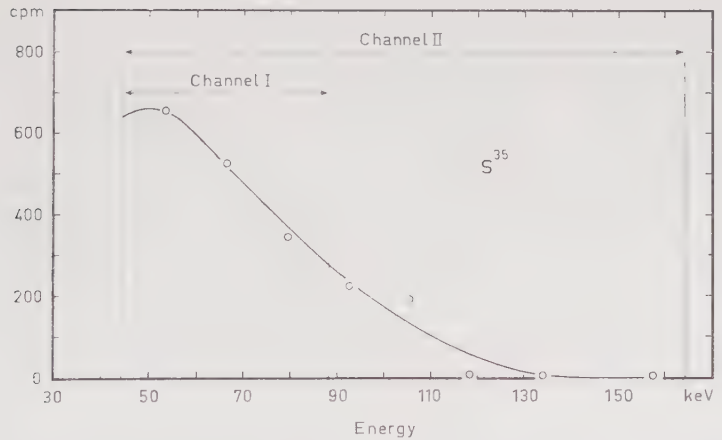

Figure 6. The channel settings used in the analysis of C^{14} and S^{35} by liquid scintillation counting. The abscissal energy values are only approximate.

7. The presence of carriers

Good reproducibility in the analysis of C^{14} is obtained, because adsorption to glass walls, platinum net and wires is avoided, since relatively large quantities of carbon dioxide and water arise from inactive material in the burning process.

In the combustion of sulphur-containing substances sulphur dioxide and sulphur trioxide are formed. Addition of inactive sulphates resulted in very varying recoveries of the radioactive isotope. This may be because the sulphur oxides formed do not react chemically with the absorption solution, but are only dissolved in the small amount of water formed during combustion. If much sulphur is present the amount of water formed may be insufficient to dissolve all the sulphur oxides formed. Therefore in the analysis of S^{35} in the tissue samples no inactive sulphur compound was used as a carrier.

In order to investigate whether there was any adsorption of S^{35} to the platinum wire and platinum net after the burning of a S^{35}-sulphate sample, these were placed in absorption solution for 1–2 hours, after which the whole absorption solution was analysed for S^{35}. No difference was found between these samples and ordinary background samples.

D. SOLUTIONS

Normal Ringer solution: 6.5 g NaCl (111.2 mM), 0.2 g KCl (2.68 mM), 0.2 g $CaCl_2$ (1.80 mM), 0.1 g $NaHCO_3$ (1.19 mM) and 1 g glucose (5.6 mM) per litre H_2O (pH = 7.2).

Radioactive Ringer solutions were obtained by the addition of $Na^{22}Cl$, $Na^{24}Cl$, $K^{42}Cl$, NH_4Br^{82}, NaI^{131} or $Na_2S^{35}O_4$ to the normal solution. Na^{24} and K^{42} were obtained as $NaHCO_3$ and K_2CO_3, which were converted to chlorides with HCl.[1] Br^{82} was obtained by neutron radiation of NH_4Br and was dissolved in distilled water.[1] S^{35} was obtained by a (n, p) reaction in the form of carrier-free $Na_2S^{35}O_4$ in aqueous solution.[2]

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[1] These isotopes were obtained from AB Atomenergi, Stockholm, Sweden.

[2] Na^{22}, S^{35} and C^{14}-labelled carbohydrates were obtained from The Radiochemical Centre, Amersham, England.

In studies of the distribution of C^{14}-labelled carbohydrate molecules, C^{14} was added to Ringer solution in the form of C^{14}-mannitol ($OHCH_2(CHOH)_4\ C^{14}H_2OH$), C^{14}-sucrose (U) or C^{41}-maltotriose (U). The two latter substances were uniformly labelled. No check of the radiochemical purity was made immediately prior to use.

All radioactive material was added to the normal Ringer solution.

The quantity of radioactive material injected into the animals is given when the experimental data are presented.

CHAPTER III

Total sodium and potassium content in the cardiac and skeletal muscle

The distribution of sodium and potassium in different tissues has long been the object of great interest. Since these ions are considered to be intimately associated with impulse formation and propagation in all excitable tissue (see further introduction to part II), interest has also been centred on their distribution in the heart. DAVIES *et al.* (1952) found in the ox heart a very high sodium concentration in the conduction system, a lower value in the atrium and the lowest in the ventricle, while the distribution of potassium was the reverse of that of sodium. MAZEL and HOLLAND (1958) reported similar findings in the frog heart.

The aim of the experiments reported here was to determine the distribution of electrolytes between the intra- and extracellular spaces in different regions of the heart, and to compare this with the electrolyte distribution in skeletal muscle.

It was first necessary to determine the ECV and the total content of sodium and potassium in the different parts of the heart, in skeletal muscle and in the plasma. The sodium and potassium contents in biological tissues can be determined in several ways, e. g. by analysis of the stable isotopes or with the aid of radioactive isotopes. One difficulty with regard to the use of radioactive potassium isotopes in the determination of the total potassium in cardiac and skeletal muscle tissue is that potassium requires a very long equilibration time, since it is mainly intracellular, and much of this exchanges very slowly with the extracellular potassium (WEATHERALL, 1962 *a*). WEATHERALL (1962 *b*) found that in the rabbit atrium approximately 20 % of the potassium is exchanged very slowly.

and that the major part of this was localized to the sarcosomes. Since only short-lived radioactive potassium isotopes are available, these decay to too low activities before complete equilibration has been attained, and recourse must be had therefore to stable potassium. Analysis of stable sodium was also made.

METHODS

Toads were used for the experiments, these being kept as described in chapter II. (With regard to the significance of the periods of captivity, see below.)

The dissection of the toads, the collection of blood and other tissue samples, combustion and other sample preparations and the final determination by flame photometry were performed as described in chapter II.

RESULTS

The results have been summarized in the accompanying tables. Table 2 summarizes the determinations of sodium and potassium in the plasma. No definite difference in values was found between toads which had been kept in captivity for different lengths of time, and the table shows only the combined results. Table 3 shows the sodium and potassium content in the heart and skeletal muscle of toads after different periods of captivity. It may be seen that the sodium content in the different tissue preparations remains on the whole constant, except for the skeletal muscle where the sodium content increases significantly after long periods of captivity ($P < 0.01$ on comparison of newly captured toads with toads captured 6–8 months previously). The potassium content is significantly lower ($P < 0.01$) in all tissue preparations after 6–8 months captivity than in newly captured toads.

Table 2.

Sodium and potassium concentrations in toad plasma (*Bufo bufo*) regardless of period of captivity. The tables show the mean values ± one standard deviation, the variation range and the number of determinations.

	meqv/l	Variation range	No. of determinations
Sodium	122 ± 4.8	110 — 133	50
Potassium	3.1 ± 0.6	1.8 — 4.5	40

Table 3.

The sodium and potassium concentrations in the cardiac and skeletal muscle after varying periods of captivity. The table shows the mean values, standard deviation and number of determinations.

Preparation	SODIUM Meqv/kg wet tissue	No. of determinations	POTASSIUM Meqv/kg wet tissue	No. of determinations
Toads, in captivity < 3 months.				
Sinus venosus	77.4 ± 10.3	14	24.5 ± 2.0	14
Atrium	62.3 ± 7.8	14	35.7 ± 5.4	14
Ventricle	37.9 ± 5.5	14	59.5 ± 5.8	14
Skeletal muscle	24.2 ± 3.5	20	72.3 ± 3.7	20
Toads, in captivity approx. 6 months.				
Sinus venosus	81.0 ± 10.2	5	20.5 ± 1.2	4
Atrium	63.7 ± 8.9	8	25.9 ± 9.8	8
Ventricle	35.0 ± 4.4	10	35.2 ± 6.1	10
Skeletal muscle	26.0 ± 7.2	9	54.1 ± 7.5	10
Toads, in captivity approx. 8 months.				
Sinus venosus	67.1	3	13.9	3
Atrium	67.5 ± 14.0	6	19.7 ± 5.3	6
Ventricle	39.8 ± 5.2	6	34.0 ± 6.4	6
Skeletal muscle	41.0 ± 5.7	6	43.8 ± 2.1	6

Table 4.

The ratio of Na/K in different heart regions after varying periods of captivity.

Preparation	Mean value	Variation range	No. of determinations
Toads in captivity <3 months.			
Sinus venosus	3.2	2.9 — 3.7	14
Atrium	1.7	1.6 — 2.0	14
Ventricle	0.6	0.5 — 0.9	14
Skeletal muscle	0.3	0.3 — 0.5	20
Toads, in captivity approx. 6 months.			
Sinus venosus	3.8	3.5 — 4.1	4
Atrium	2.8	1.8 — 4.9	8
Ventricle	1.0	0.8 — 1.5	10
Skeletal muscle	0.5	0.3 — 0.9	9
Toads, in captivity approx. 8 months.			
Sinus venosus	4.9	4.5 — 5.7	3
Atrium	3.9	2.1 — 6.8	6
Ventricle	1.2	0.9 — 2.0	6
Skeletal muscle	0.9	0.8 — 1.1	6

Table 4 gives the Na/K ratios (of molar concentrations) in the different tissue preparations after varying periods of captivity. For all tissue preparations the ratio increases after a long period of captivity, this being due mainly to a decreased potassium content.

DISCUSSION

The results obtained on the sodium and potassium content in the atrium and ventricle conform well with earlier findings (Jojima and Kuriyama, 1957). The electrolyte content in the tissues of frogs and toads varies greatly with the time of year and the length of time which the animals have been kept in captivity (Pórszász *et al.*, 1962). As may be seen in Table 3, the tissue potassium in particular alters. A possible explanation for this change may be variations in the general condition of the animals or their metabolism during captivity. The temperature at which they are kept (8–14°C) may also be of some significance to the electrolyte composition in the tissues. It is known that tissues *in vitro* that are subjected to low temperatures lose potassium (Hashish, 1958; Eliassen and Leivestad, 1961). In this investigation, however, no investigation of the body temperature of the toads in relation to the ion concentrations was made.

Possible explanations for the variations in the electrolyte composition of the tissues

Since sodium is mainly extracellular, a tissue with a large extracellular space should have a high sodium content.

The sinus venosus contains more sodium per unit weight than the atrium and ventricle. This may be an expression of the fact that the sinus venosus contains more connective tissue than the atrium and ventricle, since connective tissue is known to contain more sodium than muscle tissue (see Davies *et al.*, 1952, Kisch, 1961, *inter alia*). A second possible explanation may be that the sinus venosus and the conduction system contain a larger quantity of extracellular water than the atrium and ventricle. A third possibility is that the conduction system has

a different intracellular ion composition than other tissues, e. g. atrial muscle, and that there is a continual transition between the ionic compositions of the pacemaker-conduction system and that of the skeletal muscle. The latter tissue has no pacemaker properties, and furthermore has a completely different ionic composition from the sinus venosus. The atrium and ventricle are latent pacemakers. These tissues have an ionic composition that lies midway between that of the conduction system and that of the skeletal muscle.

To assess the last alternative, the intracellular sodium and potassium concentrations had to be determined in the different tissues. These results are reported in chapter VII.

CHAPTER IV

The chloride content and the halide spaces in the cardiac and skeletal muscle

The distribution of the anions in the tissues has not received as much attention as that of the cations, since it has been considered that anions are passively distributed and do not thus contribute to the resting potential to any noteworthy degree. One difficulty with regard to chloride ions in different tissues has been that of finding a satisfactory method of analysis. These factors are in part at least responsible for the paucity of information regarding the distribution of the chloride ion. The data in the literature on the intra- and extracellular chloride contents and chloride distribution in cardiac and skeletal muscle tissue vary greatly from author to author, depending on which method has been used in analysing the chloride, and which substance has been used for the determination of the extracellular volume.

The analysis of chloride in biological material especially in tissues, which has been a difficult problem, comprises two principal operations: 1. The preparation of a solution which contains *all* chloride in the tissue sample but no substances which interfere with the final determination; 2. The measurement of chloride in the solution by means of a colorimetric, titrimteric or other method.

The most commonly used preparation method has been wet charring with concentrated nitric acid (VAN SLYKE, 1923), followed by Volhard titration (see, for example, KOLTHOFF and SANDELL, 1961). Since this wet charring method has given doubtful or apparently erroneous results, it has been modified repeatedly. The modifications have been compared directly with other methods on series of tissue samples, but the results have varied greatly. For example chloride values obtained by

a dry charring method were the same as those obtained with VAN SLYKE's wet charring method (1923) in one comparison (CASTER, 1955), but were higher in another (CHEEK and WEST, 1955). Methods in which extracts of fresh muscle tissue with warm water or diluted sodium nitrate solution were used, gave chloride values which were about 60 % higher than the values obtained by VAN SLYKE's method (HEILBRUNN and HAMILTON, 1942).

The disparities in the chloride results obtained by different methods have been greatest in analyses of liver and muscle, which have a low content of chloride in relation to protein and other organic substances. As a rule it has been assumed that the method which gave the higher results was the more correct one, and the lower results were considered to be due, *inter alia*, to inadequate extraction of chloride or loss during the wet or dry charring process (COTLOVE, 1963 *a*).

Some of the methods available for chloride determination in biological material have fulfilled the ordinary demands of accuracy, i. e. reproducibility, complete recovery of the chloride added and agreement of the results with those of another method. These criteria do not preclude errors, however. COTLOVE (1963 *a*) got round the difficulties introduced by possible incomplete chemical recovery by making use of the isotope dilution principle. A known quantity of Cl^{36} with a known specific activity was mixed with the biological sample. This was burned, and it was assumed that there was complete mixing between stable and radioactive chloride. The true chloride content in the biological sample could then be calculated by determining the reduction in the specific activity of the tracer due to dilution with the stable chloride in the sample. In isotope dilution analysis it is of course necessary that the final chemical determination method is accurate. COTLOVE (1963 *b*), using the isotope dilution method as a reference, compared other chemical analyses of chloride in tissue.

The values which COTLOVE (1963 *a*, *b*) found for the chloride content in the muscle and liver were lower than most of the previous results. Some of the values reported in the literature differed by no more than 20 % from those obtained by the isotope dilution method, while others deviated much more.

For the analysis of biological fluids, direct dilution and titration were found to be accurate procedures.

The variation in the chloride content in many tissue samples can occur through differences in the condition of the animals, the site from which the sample is taken and varying contents of such tissue components as blood, interstitial fluid and connective tissue. While such variations may explain differences of up to $\pm$ 20 % (COTLOVE, 1963 *a*) in comparisons between tissue samples from different animals, the larger variations must be ascribed to errors in the analytical methods.

As a rule the intracellular concentrations of chloride are lower than the extracellular. Several different theories have been advanced to explain this asymmetric chloride distribution in muscle tissue. According to one theory (FENN, 1936; HASTINGS and EICHELBERGER, 1937) the muscle membrane *in vivo* is completely impermeable to chloride ions. It has been shown under *in vitro* conditions, however, that the muscle membrane is permeable to chloride ions (BOYLE and CONWAY, 1941; ADRIAN, 1960, 1961; HARRIS, 1960).

According to another theory (BOYLE and CONWAY, 1941, and others) the muscle membrane *in vivo* is *slightly* permeable to chloride ions, which are distributed completely passively under the electrochemical forces, i. e. there is a Donnan distribution. According to this concept the ratio for the extra- and intracellular chloride concentrations, $[Cl]_e/[Cl]_i$ is determined by the resting potential, which is normally considered to be the greater part of a diffusion potential for potassium ions.

A third theory (YANNET and DARROW, 1940; HEILBRUNN and HAMILTON, 1942; SHENK, 1950) suggests that there is some amount of nondiffusible (very slowly exchanging) or bound chloride, which is localized intra- or extracellularly. According to SIMON *et al.* (1957), TROSHIN (1958) and LING (1960) a certain degree of intracellular binding of monovalent ions occurs, but not definitely of chloride ions only. These hypotheses have not received strong experimental support, however.

KEYNES (1963) found in squid axons that the chloride distribution was not in accordance with that expected from a passive distribution between the axoplasm and the extracellular fluid. The determination of the activity coefficient in the axoplasm

showed that this was almost the same as for chloride in free solution, which appears to preclude the concept that the chloride is bound inside the axon to any great extent.

Some investigations have also been made on the chloride distribution in the heart. LAMB (1961) showed that the intracellular concentration of chloride in rat atria was 19.5 meqv/kg water, while the plasma chloride concentration was 108 meqv/kg. The equilibrium potential calculated by putting these values into the Nernst equation was 47 mV.

The chloride content in different regions of the heart is still very uncertain. Furthermore the data on the intracellular chloride concentration vary considerably. The aim of the present investigation was therefore to determine, by means of a method which seemed reliable, the chloride content in different regions of the heart and in skeletal muscle, and to calculate the chloride spaces. A further aim was to compare the distribution of different halide ions in the heart. This is of some interest, since, for example, bromide ions are often used instead of chloride ions in distribution and ionic flow studies in tissue preparations, and in ECV determinations.

METHODS

In a series of 10 toads the chloride content was determined in different regions of the heart, in skeletal muscle and also in plasma. The experiments were performed on newly caught toads. The dissection of the toads, the collection and preparation of samples, and the determinations were performed as described in chapter II.

Determination of Br^{82}-bromide and I^{131}-iodide spaces in the heart and skeletal muscle

In order to obtain an idea of the bromide and iodide spaces in different regions of the heart and in skeletal muscle, approximately 1 ml radioactive Ringer solution, containing about 10 μC Br^{82} (specific activity: 74 mC/g Br) and 10 μC I^{131} (carrier-free), was injected into the posterior lymph sac of nine toads, after which these radioactive isotopes were allowed to distribute for about 30 hours. Tissue and blood samples were

then taken as described in chapter II. The plasma samples were transferred quantitatively to analysis tubes, as also were the heart and muscle preparations, and analysed for their content of Br^{82} and I^{131} by means of gammaspectrometry (see chapter II, page 21).

Determination of proteins in plasma

The plasma proteins were determined in a series of 10 toads, as described in chapter II, page 19.

RESULTS

The chloride content in heart and muscle tissue

The results of the chloride determinations in heart and skeletal muscle in toads (*Bufo bufo*) are shown in Table 5. It may be seen in the table that the content of chloride in the sinus venosus is greatest, followed by the atrium and then the ventricle. The heart as a whole has a higher content than skeletal muscle.

The chloride content in plasma

The mean chloride concentration in the plasma from 10 toads was 91.3 ± 5.5 meqv/litre (± 1 S. D.).

The halide spaces in the heart and skeletal muscle

The volume of water in the extracellular space in a sample can be calculated if the total quantity and concentration of a substance, which is known not to penetrate the cell membrane,

Table 5.

The chloride content in the heart and skeletal muscle of newly caught toads (*Bufo bufo*). The table shows the mean values ± one standard deviation.

Preparation	meqv Cl^- per kg wet tissue	No. of determinations
Sinus venosus	72.0 ± 7.2	5
Atrium	47.2 ± 4.6	10
Ventricle	23.8 ± 1.4	9
Sartorius muscle	15.0 ± 1.5	10

is determined. Several substances have been used for this purpose. It has been assumed here provisionally that chloride, bromide and iodide ions are distributed only in the extracellular space. This is not entirely correct since a certain fraction of these ions do enter the cells, but a calculation based on the assumption that they are entirely extracellular give some idea of the extracellular space, and may be of some guidance in comparisons of the size of this space in different heart regions and in skeletal muscle.

Calculation of the results

The calculation of the different spaces refers to 100 g wet tissue. The following notation will be used:

$[H_2O]_p$ = ml water per litre plasma
$[Cl]_p$ = chloride concentration in plasma (meqv/l)
$[Br^{82}]_p$ = Br^{82} concentration in plasma (cpm/l)
$[Cl]_e$ = chloride concentration in the extracellular water (meqv/l)
$[Br^{82}]_e$ = Br^{82} concentration in the extracellular water (cpm/l)
$[Cl]_m$ = meqv chloride per 100 g wet tissue
$[Br^{82}]_m$ = cpm Br^{82} per 100 g wet tissue

The plasma water was not determined but was calculated on the basis of the protein concentration according to the equation below, given by Eisenman, Mackenzie and Peters (1936), and tested for protein concentrations from about 3 to 8 g/100 ml plasma.

$$[H_2O]_p = 984 - 7.18 \cdot \text{protein concentration (in g/100 ml plasma)}.$$

The mean protein concentration in plasma from 10 toads was 2.6 ± 0.5 g/100 ml plasma (duplicate samples from each toad), which gives a water concentration of 965 g/l plasma. The protein concentration in the toad plasma is low compared with the mean value of 7.5 g/100 ml plasma in man (Bergström, 1962). In comparison it may be mentioned that rats have a plasma protein concentration of 3.5 g/100 ml plasma (Rauen, 1956).

On calculating the chloride concentration in the extracellular water, consideration should perhaps be taken of the probable

existence of a Donnan equilibrium for the diffusible ions between plasma and the extracellular space. Since plasma contains proteins, which, it is assumed, do not enter the extracellular space, the concentration of chloride in the extracellular water should be higher than in the plasma. In calculations in man, a Donnan ratio (r) of 0.96 has been used (see VAN SLYKE, 1923 and BERGSTRÖM, 1962). In calculations on the toad the Donnan factor will be less important, since the plasma protein content is so low. The permeability conditions in toads, may be different from those in mammals, and since neither the plasma pH, the isoelectric points of the plasma proteins nor the protein fractions themselves were investigated it is difficult to correct for a Donnan distribution.

If a Donnan ratio is used, the following equation holds:

$$[Cl]_e = \frac{[Cl]_p \cdot 1000}{r \cdot [H_2O]_p}$$

If no Donnan factor is used r will be put equal 1.

The extracellular Br^{82} and I^{131} concentrations were calculated in the same way. In doing this it was presupposed that Br^{82} and I^{131} were distributed similarly to chloride ions with the same Donnan ratio.

In the calculations correction was made for the solid content of plasma.

The chloride space was calculated according to the following formula:

$$\text{The chloride space} = \frac{[Cl]_m \cdot 100}{[Cl]_e} \text{ ml/100 g wet tissue.}$$

The Br^{82}-bromide and I^{131}-iodide spaces were calculated similarly.

The chloride, bromide and iodide spaces in different tissues

In these calculations no Donnan correction was used, since this factor is difficult to determine. The mean values for chloride space determinations in toads (*Bufo bufo*) are shown in Table 6. The preparations consisted of sinus venosus, atrium, ventricle and skeletal muscle (sartorius).

Table 6.

The chloride spaces in the heart and skeletal muscle of the toad (*Bufo bufo*). The table shows the mean values ± one standard deviation.

Preparation	ml per 100 g wet tissue	No. of determinations
Sinus venosus	76.6 ± 7.7	5
Atrium	50.3 ± 5.8	10
Ventricle	25.4 ± 2.1	9
Sartorius muscle	16.0 ± 1.4	10

The results of the simultaneous bromide and iodide space determinations may be seen in Table 7. The results are the mean values from nine toads (*Bufo bufo*). Fig. 7 shows the relationships between the different halide spaces in the heart and skeletal muscle. The bromide and iodide spaces were determined simultaneously, while the chloride spaces were determined on other toads.

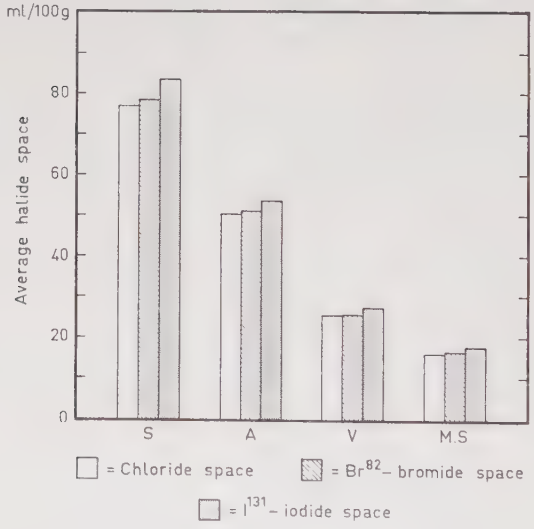

Figure 7. Mean values of halide space determinations (ml/100 g wet tissue) in heart and skeletal muscle. Br^{82}-bromide and I^{131}-iodide spaces determined simultaneously. Chloride spaces determined on other toad caught at the same time period.
S = sinus venosus. A = atrium. V = ventricle. M.S. = sartorius muscle.

Table 7.

The Br^{82}-bromide and I^{131}-iodide spaces in the heart and skeletal muscle of newly caught toads (*Bufo bufo*). The determinations were made simultaneously. The table shows the mean values ± one standard deviation.

Preparation	Br^{82}-bromide space		I^{131}-iodide space	
	ml per 100 g wet tissue	No. of determinations	ml per 100 g wet tissue	No. of determinations
Sinus venosus	78.4 ± 2.3	9	83.6 ± 5.2	8
Atrium	51.1 ± 6.6	9	53.5 ± 6.5	9
Ventricle	25.4 ± 2.6	9	27.3 ± 1.9	9
Sartorius muscle	16.5 ± 2.4	9	17.6 ± 1.6	9

DISCUSSION

Determination of chloride in tissue

The method was found to give reproducible values. In burning the preparation it was probable that all chloride became available for analysis, including that situated intracellularly. The method has not, however, been compared with any other method, e. g. the isotope dilution method, and the possibility cannot therefore be precluded that some chloride may have disappeared during the actual preparation of the samples. By burning the samples (including the plasma samples) any interference of proteins on the mercurimetric titration was avoided. Phosphate can influence the end point of the mercurimetric titration, but Danielson (1963) found that this effect does not hold at pH = 3.5, and he also showed that there was a 100 % recovery of the amount of chloride added. In preparing the samples it is important that a large excess of calcium acetate solution is added and this is allowed to evaporate slowly in order to avoid chloride losses during the burning. Losses may have occurred during the sample preparation and burning, but since the values for the different halide spaces are in such good agreement it is improbable that any large loss occurred during the sample preparation.

The physico-chemical condition of chlorine in the tissue

Chlorine in the tissues is probably mainly in the ionized form. This ion has a very weak affinity for proteins. There

are a few reports that chloride ions bind to proteins in biological fluids and tissues (PETERS and VAN SLYKE, 1931), and binding to plasma proteins is presumably small, owing to competition with other substances with a higher affinity for proteins, e. g. fatty acids, amino acids, organic acids and other proteins (COTLOVE and HOGBEN, 1962).

Evidence of protein binding of chloride has only been obtained in relatively simple systems, which have contained a purified protein and a single salt (KLOTZ, 1953). It has been shown that the binding affinity of chloride to serum albumin is approximately 1/4 of that of iodide. Serum globulin binds less chloride per unit weight than serum albumin (CARR, 1953).

It is thus probable that chloride exists mainly as a free diffusible ion.

The chloride content in different tissues

The chloride concentration varied in different regions of the heart, with the highest value in the sinus venosus, followed by the atrium and ventricle in that order. The average chloride concentration in the whole heart was higher than that of the sartorius muscle. Chloride has principally the same distribution as sodium (see chapter III), but the total molar concentration of sodium is higher than that of chloride in both the heart and skeletal muscle, in toads caught at the same time of year (cf. Table 8). It is concluded that the greatest part of the sodium chloride in the heart and skeletal muscle is extracellular, since in animal experiments only a very small part has been found

Table 8.

The ratio of Na/Cl (of molar concentrations) in the heart, skeletal muscle and plasma of newly caught toads (*Bufo bufo*). The table shows the mean values ± one standard deviation (calculated as the standard deviation for a ratio).

Preparation	Na/Cl
Sinus venosus	1.1 ± 0.2
Atrium	1.3 ± 0.2
Ventricle	1.6 ± 0.3
Sartorius muscle	1.6 ± 0.4
Plasma	1.3 ± 0.1

to exchange slowly (AMBERSON *et al.*, 1938), and since radioactive chloride and sodium ions penetrate the entire chloride and sodium spaces in rat heart in less than 8 minutes (MANERY and HAEGE, 1941; MANERY and BALE, 1941).

It may be assumed that part of the electrolytes are in connective and fat tissue. It has been reported in dogs that about 30 % of all chloride in the muscle is in connective tissue, and about 15–20 % in the rat and cat muscle (COTLOVE and HOGBEN, 1962). The greatest part of this chloride should be easily exchangeable (AMBERSON *et al.*, 1938; WOODBURY, 1956), while about 2 % of the total chloride in cat muscle exchanges slowly (COTLOVE and HOGBEN, 1962). According to MANERY (1961) loose connective tissue has approximately the same chloride concentration (related to the tissue water) as plasma. According to LOWRY, GILLIGAN and KATERSKY (1941) the connective tissue constitutes a small but constant part of the heart and skeletal muscle tissue, and the quantity of electrolytes in this tissue is therefore probably small compared with the quantity localized in the remaining part of the extracellular spaces of muscle tissues. It is known from histological evidence that connective tissue is relatively abundant in the atrium and the sinus venosus, and thus the total electrolyte content of these tissues may therefore be relatively large. Any sodium and chloride which is bound in the connective tissue must, however, be easily exchangeable, since the sodium and bromide spaces very rapidly reach their maximal values (cf. chapter IX).

According to MANERY (1961), fat tissue also has approximately the same chloride concentration as plasma (in terms of tissue water). COTLOVE (1963 *a*) found that neutral fat contained only 0.3 % of the total chloride content in the skeletal muscle of the rat. As is evident in chapter II the fat extractable with petroleum ether is a very small part of the wet weight in the tissues investigated.

Comparisons between different halide spaces in the same tissue

The extracellular volume in muscle as estimated by the relative area of the space between the fibres in histological

sections corresponds approximately to the chloride space, and was one of the reasons that the chloride space became used as an approximation for extracellular space (FENN, 1936). Stable or radioactive bromide have been used similarly for the determination of bromide spaces in different tissues. The distribution of bromide greatly resembles that of chloride in most tissues (WEIR and HASTINGS, 1939; WALLACE and BRODIE, 1939; GAMBLE *et al.*, 1953).

The results of the space determinations for the different halides show that these do not definitely differ significantly (in all cases $P > 0.02$) for any particular tissue. Despite certain physico-chemical dissimilarities between the ions (see further chapter VII), the distribution appears on the whole to be the same for the tissues investigated here.

Comparisons between halide spaces in different tissues

There are several possible explanations for the differences in the size of the halide spaces between the different tissues (sinus > atrium > ventricle > skeletal muscle). If, firstly, the halide spaces are assumed to be approximations for the extracellular space, the variations in size of the spaces should indicate that the size of the extracellular space varies in different regions of the heart and that the heart has a larger extracellular space than the skeletal muscle. Secondly, the intracellular halide concentration may vary in different tissues. In order to assess in more detail these possibilities studies have been made on the distribution of other substances, which to the greatest part are distributed in the extracellular space. With the aid of these "extracellular space approximations" it is possible to assess more accurately the size of the extracellular space and also calculate the intracellular electrolyte concentrations if the total electrolyte content of the tissue and the plasma concentrations are known.

For a discussion of the *length of the distribution time*, see further chapter VII.

CHAPTER V

The extracellular space in the heart and skeletal muscle

The electrolyte content of the intracellular phase must be calculated as the difference between the total tissue electrolyte and the electrolytes present in the extracellular space (water and connective tissue). For determination of the latter the volume of the extracellular space must be known, its electrolyte concentration being calculated from the corresponding plasma values. Correct evaluation of the extracellular space is especially important in the case of such ions as sodium and chloride whose intracellular content is low. .

The extracellular space in a tissue sample is conventionally calculated from the total tissue content of a molecular or ionic species which is regarded as being distributed only throughout the extracellular space without entering the specific cell phase (e. g. muscle fibres). There appears to be no substance ideal for this purpose. The extracellular indicator should have the following properties: (a) it must not be metabolized, (b) it must not enter the specific cells, i. e. it must be too large or have a structure preventing this and (c) it must distribute throughout the solvent in the extracellular space as in plasma, since its concentration in the latter is used as the reference value. This means that it should not become fixed (e. g. adsorbed) in any extracellular site. The extracellular space is, however, a complex structure containing in addition to cellular elements various solid structures, i. e. fibrillar (see chapter I), and also the high polymer hyaluronic acid so that its physicochemical properties are complicated (Meyer *et al.*, 1956; Rogers, 1961; Ogston and Sherman, 1961). The choice of an extracellular indicator is therefore somewhat arbitrary and

often a non-electrolyte, e. g. mannitol or inulin, is chosen. These molecules are highly water soluble, neutral and are not known to undergo any specific interactions in the extracellular space. As mentioned in chapter I, various electrolytes resembling the chloride and sodium ions in their distribution have also been used for this purpose.

The aim of this investigation was therefore to study the distribution of certain substances, which it is believed remain essentially extracellular, and to compare their calculated distribution spaces with the sodium and halide spaces.

METHODS

The distributions of C^{14}-mannitol, C^{14}-sucrose, C^{14}-maltotriose (three 1–4 α linked glucose units) and S^{35}-sulphate in the form of carrier-free $Na_2S^{35}O_4$ were studied in different series of toads kept for 5–7 months since capture. Approximately 1 ml normal Ringer solution containing about 5 μC of the radioactive substance was injected into the posterior lymph sac of each toad, after which the labelled solutes were allowed to distribute for different lengths of time (15 mins to 50 hours). At the end of the distribution period the toads were pithed and tissue and blood samples were collected, after which the plasma and tissue samples were prepared and analysed for their radioactive contents as described in chapter II.

The different spaces were calculated in terms of 100 g wet tissue in the same way as for the halides spaces (cf. chapter IV, page 40), using also the same values for the water and protein content of plasma (see page 40).

RESULTS

The C^{14}-mannitol spaces

Figs. 8 and 9 show the C^{14}-mannitol spaces (mol wt = 182) in the heart and skeletal muscle of the toad in relation to the distribution time. The space in the sinus venosus is of the order of 60 ml/100 g tissue after distribution periods of 0.5–10 hours, but increases after longer distribution periods to about 70 ml. The mannitol space in the atrium is about 40 ml/100 g tissue after distribution period of 1/2–10 hours, reaching

after about 35 hours values about 55 ml/100 g tissue, i. e. of the same order as the Br^{82}-bromide space (cf. chapter IV, page 43 or chapter VI, page 60). This progressive increase probably means that the mannitol slowly penetrates the cell. In the ventricle the mannitol space is smaller than of Br^{82}, up to a distri-

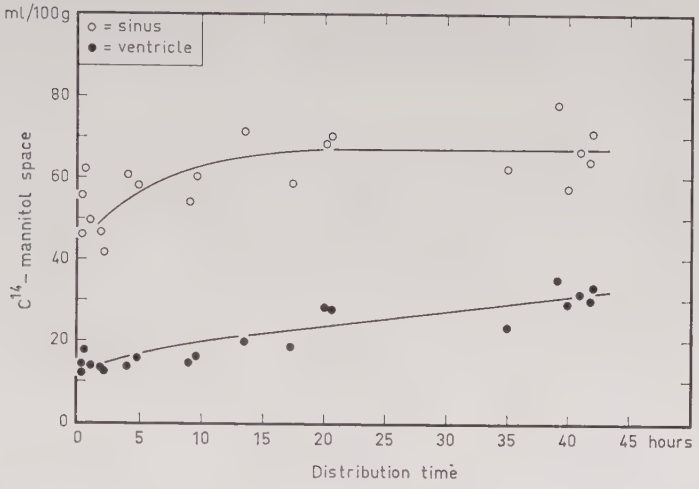

Figure 8. C^{14}-mannitol spaces (ml/100 g wet tissue) in the sinus venosus and ventricle after different distribution periods. (The curves are drawn by eye).

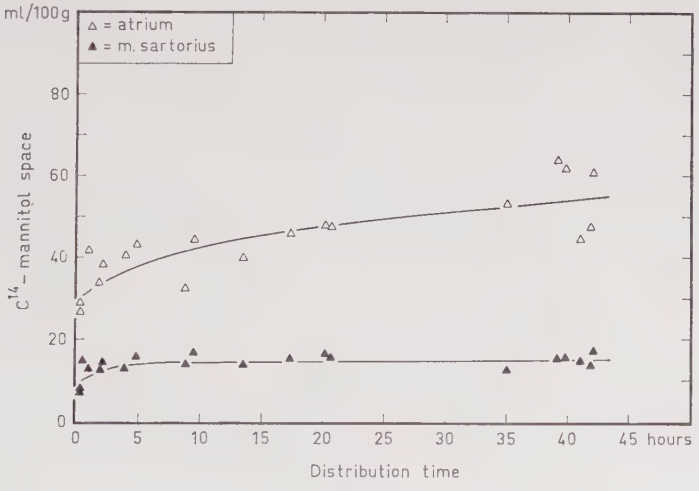

Figure 9. C^{14}-mannitol space (ml/100 g wet tissue) in the atrium and sartorius muscle after different distribution periods. (The curves are drawn by eye).

bution period of about 20 hours, but after longer periods increases considerably, reaching values of about 35 ml/100 g wet tissue after a distribution period of 40 hours, compared with 15 ml after a shorter period (0.5–10 hours). For skeletal muscle (sartorius) the mannitol space is about 14 ml/100 g wet tissue, and remains the same even after as long a distribution period as 40 hours. Table 9 shows the C^{14}-mannitol determinations where the radioactive substance was allowed to distribute for 0.5–9.5 hours.

Table 9.

The C^{14}-mannitol spaces in the heart and skeletal muscle of the toad (*Bufo bufo*). The table shows the mean values ± one standard deviation for determinations where C^{14}-mannitol had distributed for 0.5 — 9.5 hours, and also the number of determinations.

Preparation	ml/100 g wet tissue	No. of determinations
Sinus venosus	54.2 ± 7.5	8
Atrium	39.1 ± 4.6	7
Ventricle	14.7 ± 1.8	8
Sartorius muscle	14.2 ± 1.4	8

The C^{14}-sucrose spaces

Figure 10 and Table 10 show the results of the determinations of the C^{14}-sucrose spaces (mol wt = 342) in the heart and skeletal muscle. Determinations were made on six toads. For the sinus venosus a mean value of 61.1 ml/100 g wet tissue was obtained for five determinations made with a distribution period of less than 14 hours, which is of the same order of size as the corresponding mannitol space. After a distribution period of 40 hours the sucrose space in the sinus venosus had further increased to about 75 ml/100 g tissue (one determination). For the atrium a mean value of 42.3 ml/100 g wet tissue was obtained for a distribution period of less than 14 hours, while after 40 hours the value had increased to 70 ml/100 g tissue. For the ventricle a mean value of 15.4 ml/100 g tissue (two determinations) was obtained for distribution periods of less than 4 hours, but after about 13 hours it had increased to approximately 25 ml/100 g tissue, and after 40 hours to

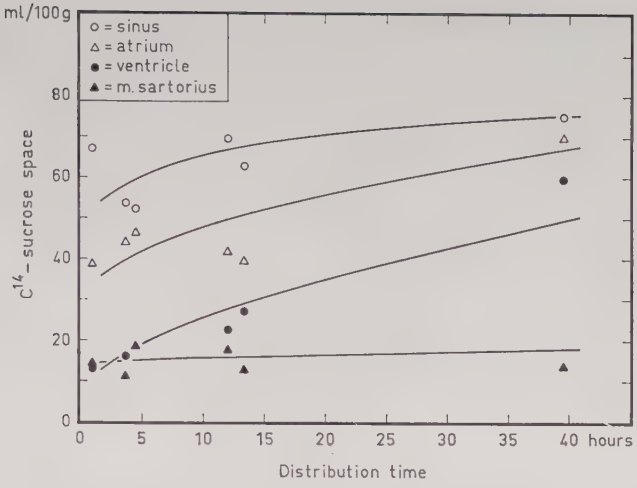

Figure 10. C^{14}-sucrose spaces in the heart and sartorius muscle (ml/100 g wet tissue) after different distribution periods. (The curves are drawn by eye).

Table 10.

The C^{14}-sucrose spaces in the heart and skeletal muscle of the toad (*Bufo bufo*). The table shows the mean value ± one standard deviation.

Preparation	ml/100 g wet tissue	No. of determinations	Distribution time in hrs
Sinus venosus	61.1 ± 7.7	5	1 — 14
Atrium	42.3 ± 3.1	5	1 — 14
Ventricle	15.4 (13.8, 16.9)	2	1, 3.5
Sartorius muscle	14.8 ± 2.8	6	1 — 39

60 ml/100 g tissue. For skeletal muscle (sartorius) a mean value of 14.8 ml/100 g wet tissue was obtained (1–14 hours) and no increase was observed even after a distribution period as long as 40 hours.

The "C^{14}-maltotriose spaces"

C^{14}-labelled maltotriose (mol wt = 504) was also used to determine the extracellular volume in the heart and skeletal muscle in the toad. Determinations were made on six toads. The results (calculated as for the other spaces, see Fig. 11) show that C^{14} is concentrated in the tissues, especially in the

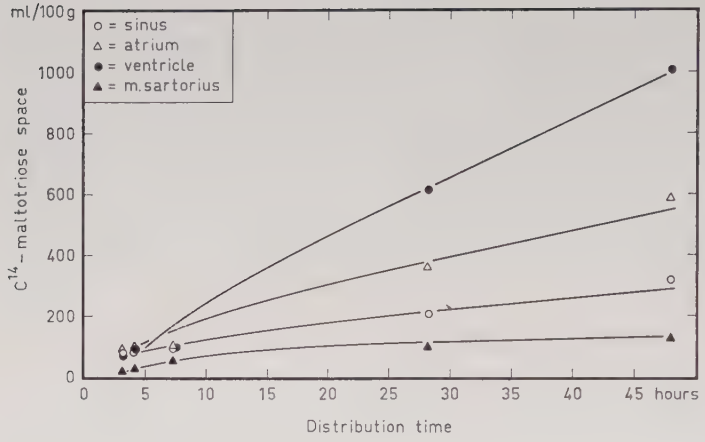

Figure 11. "C^{14}-maltotriose spaces" in the heart and sartorius muscle after different distribution periods (the results calculated analogously with other spaces). Note the scale of the ordinate.

heart, as a consequence of which the values for the "C^{14}-maltotriose spaces" are impossibly high. This is presumably due to the breakdown of the maltotriose either during storage (the maltotriose had a rather high specific activity) before use or by the tissues themselves with the result that metabolised fragments penetrated and accumulated in the cells. The greatest accumulation of C^{14} was in the ventricle, followed by the atrium and the sinus venosus. The heart as a whole had a higher content of C^{14} than the skeletal muscle.

The S^{35}-sulphate spaces

The results of the determinations of the sulphate spaces in the heart and skeletal muscle tissue are shown in Figs. 12 and 13. It may be seen that the sulphate ions rapidly distribute within a space, which then increases only insignificantly after a long distribution period. The mean values of the results of the sulphate space determinations are given in Table 11, and these values refer to all toads in which the radioactive sulphate had been distributed for more than 50 minutes. It is known that the sulphate concentration in the tissue equilibrated very rapidly (within a few minutes) with the plasma (see HUTTER, 1961; SJÖSTRAND, 1964, *inter alia*), and the rapidity with which

the maximal values were reached fit well with the hypothesis that the sulphate ions are limited to the extracellular space. It is possible, however, that a small fraction of the sulphate ions also penetrate the cell membrane, but this is so small that it can be disregarded in calculating the sulphate space.

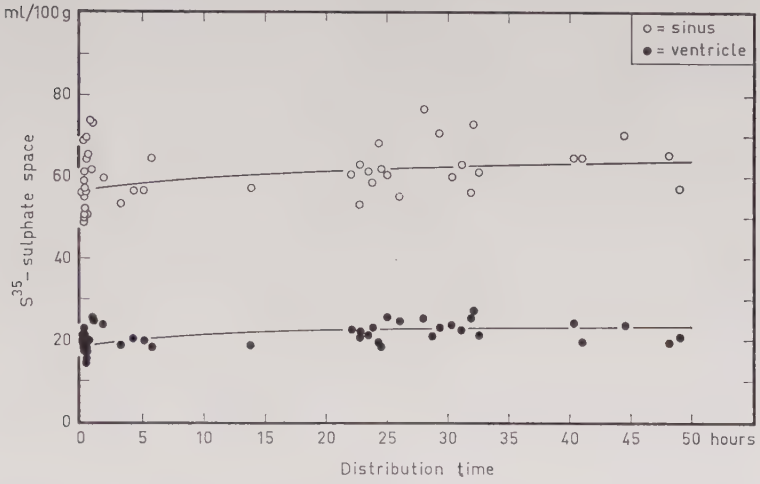

Figure 12. S^{35}-sulphate spaces (ml/100 g wet tissue) in the sinus venosus and ventricle after different distribution periods. (The curves are drawn by eye).

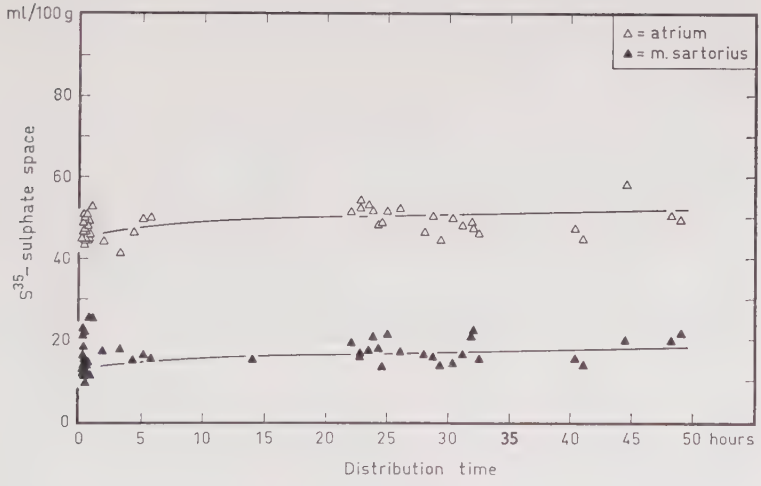

Figure 13. S^{35}-sulphate spaces (ml/100 g wet tissue) in the atrium and sartorius muscle after different distribution periods. (The curves are drawn by eye).

Table 11.

The S^{35}-sulphate spaces in the heart and skeletal muscle of the toad (*Bufo bufo*). The table shows the mean values ± one standard deviation for toads in which the radioactive sulphate was allowed to distribute for more than 50 mins.

Preparation	ml/100 g wet tissue	No. of determinations
Sinus venosus	62.8 ± 6.5	29
Atrium	48.8 ± 4.0	28
Ventricle	22.0 ± 2.5	30
Sartorius muscle	17.2 ± 2.6	28

DISCUSSION

The C^{14}-mannitol spaces

PAGE (1962) and many other authors have used the mannitol space as an indicator for the extracellular space. After short distribution periods there is nothing to indicate that the mannitol penetrates the cell membrane. The experiments reported here indicate that whereas in sinus venosus and skeletal muscle apparently steady values of the calculated space were attained (striated muscle in 5 hours, sinus venosus in 15 hours) the C^{14} activity continued to rise in atrium and ventricle over a long period ($\approx$ 45 hours). The increase was most marked in the ventricle where after about 40 hours the mannitol space was about 30 % greater than the bromide space.

The question arises as to the cause of the slow secondary increase in C^{14} activity. There seems to be no reason to doubt the radiochemical purity of the solute. Radiochromatography some months before the experiment (butanol:acetic acid:water 4 : 1 : 2 or iso-propanol:pyridine:water:acetic acid 8 : 8 : 4 : 1) indicated high purity, and gel filtration (shortly after these experiments) through a tightly cross-linked gel (Sephadex®) gave no evidence of heterogeneity (MARSDEN, *personal communication*). The gel filtration method seems to be a rather sensitive test of homogeneity provided the impurities have a different molecular weight and structure from the main component. A further indication of adequate radiochemical purity is the rapid attainment of a steady level in striated muscle. It would be hardly likely that diverse impurities were penetrant only in cardiac and not in skeletal muscle.

The shape of the radioactivity time curves indicates that at least two compartments probably exist. It seems reasonable to locate the first rapid compartment in the extracellular space. The second slow compartment may be (a) a result of hetereogeneity in the extracellular space, i. e. due either to a more slowly exchanging "solvent" region or to some fixation process such as adsorption or (b) due to entry of the solute into the intracellular space. The question of adsorption cannot be settled directly but there is no evidence that the physico-chemical properties of the tissue should provide adsorption sites for a polyhydric alcohol though considerable weak hydrogen bonding interactions may be expected. Further there is the discrepancy in behaviour between sinus venosus and striated muscle on the one hand and atrium and ventricle on the other. It seems here more reasonable to attribute these differences rather to the specific cells of the tissue than to the intercellular matrix. Thus permeation into the intracellular space appears the more likely explanation for the slow secondary rise. This finds some support in the fact that the calculated C^{14} spaces in ventricle and atrium are larger than the corresponding bromide spaces after a long distribution period. Mannitol does not appear to penetrate into striated muscle, and in view of this it is not surprising that the permeation which probably occurs in cardiac muscle should be rather slow.

The C^{14}-sucrose spaces

Uptake of C^{14} activity in all the heart tissues continued throughout the distribution periods and steady values were not obtained. Striated muscle, on the other hand, rapidly (< 5 hours) attained a steady value. The radiochemical purity was not tested, but using the striated muscle steady value as an index this seems to have been satisfactory (see Mannitol discussion). The failure of the C^{14} uptakes to reach steady values in the heart samples suggests that either the C^{14}-sucrose or some metabolic products derived from it entered the cells.

Page (1962), in *in vitro* experiments on cat heart muscle, found that the smallest molecule that could not penetrate the cell membrane was mannitol (calculated molecular radius =

= 4.0 Å) in distribution periods of up to two hours. For comparison it may be mentioned that sucrose has a molecular radius of 4.5 Å (PAGE, 1962).

From the present experiments it seems that a relatively long period is required in the heart for intracellular equilibration of C^{14}-labelled solutes in plasma, since in 40 hours the sucrose spaces had not reached values of the same order of size as the total water volume. In skeletal muscle C^{14} activity appears to remain in the extracellular space, although the possibility cannot be excluded that exceedingly slow penetration into the muscle cells does occur.

The "C^{14}-maltotriose spaces"

Since certain cells, e. g. erythrocytes (ÖSTLING, 1964), are impermeable to maltotriose (mol. wt = 504), at least *in vitro*, this substance was tested for determining the ECV in the heart, but was found unsuitable. The results (see Fig. 11) showed an accumulation of C^{14} in the heart which was especially pronounced in the ventricle. The enzyme maltase, which is found in the intestine in mammals (WHITE *et al.*, 1954) and probably also in toads, presumably splits the maltotriose with the formation of C^{14}-glucose or other derivatives, which can enter the cell either passively or actively. Penetration of maltotriose itself is hardly likely, because of the size of the molecule (PAGE, 1962).

The S^{35}-sulphate spaces

Sulphate ions have also been used for determining the extracellular space (for the heart muscle see JOHNSON, 1955; NIEDERGERKE, 1963; for skeletal muscle see WALSER *et al.*, 1954). The sulphate space has been considered to be somewhat larger than those of mannitol, sucrose and inulin. This is probably because sulphate also enters the extracellular connective tissue, in contrast to the carbohydrate molecules, which are not considered to penetrate the connective tissue (SELKURT, 1963). It is evident from the results (see Figs. 12 and 13) that the sulphate spaces rapidly reach a maximum, after which the size

remains unchanged after long distribution periods. If some of the sulphate ions do enter the connective tissue in the ECV (as claimed by SHEATZ and WILDE, 1950, among others), the cells must be very permeable to them since the sulphate space reaches its maximal value so rapidly. The specific heart cells, on the other hand, appear almost completely impermeable and if there is any sulphate penetration it must be very slow.

For more detailed comparisons between the sulphate, sodium and bromide spaces, see the discussion in chapter VI (cf. COTLOVE and HOGBEN, 1962 and WALSER *et al.*, 1954).

For a further discussion on the properties which determine the penetration capacity or permeability of cell membranes to non-electrolytes and ions, see chapter VII (discussion).

CHAPTER VI

Simultaneous determinations of the Na^{24}-sodium, Br^{82}-bromide and S^{35}-sulphate spaces in the heart and skeletal muscle

The aim of this part of the investigation was to determine the distribution of Na^{24}, Br^{82} and $S^{35}O_4$ in different regions of the heart and in the skeletal muscle *simultaneously*, in order to compare their approximate extracellular spaces. All of these ion spaces have been used in the past as approximations of the extracellular spaces in different tissues. Sodium and bromide ions penetrate the cells to some extent, and for this reason give an overestimate when used in the determination of the ECV. It is also supposed that sulphate ions enter cells (GAMBLE, 1952; cf. also chapter V), but presumably to a small extent.

In the determination of stable sodium in the different regions of the heart there are certain practical difficulties, especially with regard to the sinus venosus, but with the aid of radioactive isotopes it is possible to make an accurate determination of the total content. If the radioactive isotope Na^{24} is allowed to equilibrate with stable sodium, the total content of stable sodium can be calculated from the specific activity of Na^{24} in the plasma and the total Na^{24} content in the tissue samples (assuming that the tissue specific activity is the same as that in the plasma). The results make possible a comparison with the determinations of the Na content in the heart and skeletal muscle reported in chapter III.

METHODS

Toads (*Bufo bufo*) which had been kept in captivity for just over 7 months were used for the experiments.

Approximately 1 ml radioactive Ringer solution was injected

into the posterior lymph sac in a series of 10 toads. This solution contained 50 μC Na^{24} (specific activity 20 mC/g Na), 10 μC Br^{82} (specific activity 74 mC/g Br) and 5μC S^{35} (carrier-free) per ml. The isotopes were allowed to distribute in the toads for periods of 22–50 hours. This period can be regarded as adequate for complete equilibration of Na^{24} and Br^{82} with stable isotopes both intra- and extracellularly in the heart and skeletal muscle, since MANERY and BALE (1941) found that sodium and chloride ions equilibrated the entire chloride and sodium space in the heart in less than 8 minutes. Data for non-electrolytes which penetrate the cells indicate that isotope efflux from a previously equilibrated muscle was virtually complete in less than 100 minutes (HOROWITZ and FENICHEL, 1963).

At the end of the distribution period the toads were killed by pithing, and blood and tissue samples were collected. The sinus venosus and duplicate samples of atrium, ventricle and skeletal muscle were weighed. The tissue and plasma samples were placed on the special combustion papers used in preparation for liquid scintillation counting (see chapter II, page 25), dried in an oven at about 90°C for two hours and then analysed for their contents of Na^{24} and Br^{82} by means of gamma-spectrometry.

When about 10 half lives of Br^{82} ($T_{1/2} = 35.9$ hrs) had elapsed the samples were burned and analysed for their content of S^{35} by liquid scintillation counting (see chapter II).

Two plasma samples of 25 μl each were used for flame photometric determination of sodium (see chapter II).

RESULTS

The Na^{24}-sodium, Br^{82}-bromide and S^{35}-sulphate spaces

The results were calculated as described in chapters IV and V. No Donnan correction was used, but a correction was made for the solid content in the plasma. The results showed that there was no further increase in the size of the Na^{24}-sodium, Br^{82}-bromide and S^{35}-sulphate spaces after a long distribution period. Fig. 14 shows the mean values for the different spaces. The results clearly showed, as also did previous extracellular

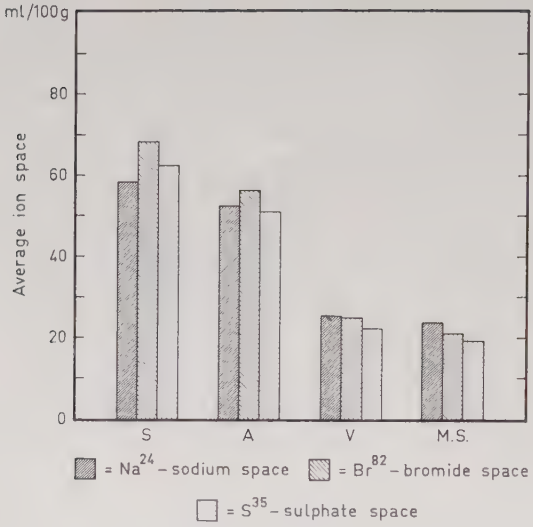

Figure 14. Mean values of simultaneous ion space determinations (ml/100 g wet tissue) in the heart and skeletal muscle.
S = sinus venosus. A = atrium. V = ventricle. M.S. = sartorius muscle.

space determinations with $S^{35}O_4$, Br^{82} and I^{131} that the sinus venosus had the largest ECV followed by the atrium, ventricle and skeletal muscle in that order, *regardless of which substance had been used for determining the ECV.*

Table 12 gives the results of the different ion space determinations. The S^{35}-sulphate spaces did not differ significantly from the Br^{82}-bromide spaces in any of the tissues ($P > 0.01$

Table 12.

The Na^{24}-sodium, Br^{82}-bromide and S^{35}-sulphate spaces in the heart and skeletal muscle in the t
(*Bufo bufo*). The determinations were made simultaneously. The table shows the mean values
one standard deviation and the number of determinations.

Preparation	Na ^{24}-sodium space		Br^{82}-bromide space		S^{35}-sulphate space	
	ml per 100 g wet tissue	No. of determinations	ml per 100 g wet tissue	No. of determinations	ml per 100 g wet tissue	No. of de minatio
Sinus venosus	58.3 ± 3.4	8	68.3 ± 3.5	8	62.5 ± 6.6	10
Atrium	52.5 ± 3.7	9	56.3 ± 5.8	9	51.0 ± 3.9	10
Ventricle	25.5 ± 3.1	10	24.7 ± 2.2	10	22.2 ± 2.7	10
Sartorius muscle	23.7 ± 2.6	10	21.0 ± 3.2	10	19.3 ± 2.1	10

in all cases). The S^{35}-sulphate space in the sartorius muscle was significantly smaller than the Na^{24}-sodium space ($P < 0.01$), but no definite difference was observed in the other tissues (for the sinus and atrium $0.5 > P > 0.1$, for the ventricle $0.05 > P > 0.02$).

The Na^{24}-sodium space in the sinus venosus was significantly smaller than the Br^{82}-bromide space ($P < 0.01$), but in the other tissues investigated there was no clear difference ($P > > 0.05$).

The total sodium content in different tissues

The specific activity of Na^{24} in the plasma was calculated on the basis of determinations of stable and radioactive sodium. The total content of sodium in the different tissues was calculated under the assumption that Na^{24} was completely equilibrated with stable sodium. The results are shown in Table 13. The values do not differ significantly from those found for stable sodium in the heart and skeletal muscle in toads which had been kept in captivity for corresponding periods (i. e. about 6 months, see chapter III, Table 3).

DISCUSSION

Ionic spaces

The fact that the Br^{82}-bromide space is larger than the Na^{24}-sodium space in the sinus venosus may be due, *inter alia*,

Table 13.

The sodium content in the heart and skeletal muscle in the toad (*Bufo bufo*), calculated on the basis of the Na^{24} content in the tissues and the specific activity of Na^{24} in the plasma. (The Na^{24} was considered to be completely equilibrated with the stable sodium). The table shows the mean values $\pm$ one standard deviation, and the number of determinations.

Preparation	meqv/kg wet tissue	No. of determinations
Sinus venosus	75.4 $\pm$ 4.0	8
Atrium	68.5 $\pm$ 6.8	9
Ventricle	33.3 $\pm$ 4.9	10
Sartorius muscle	30.7 $\pm$ 3.2	10

to the bromide intracellular concentration being higher than the sodium, or to differences in the electrolyte composition in the connective tissue, which is found in abundance in the sinus venosus (see KISCH, 1961, among others). In the other tissues investigated there were no apparent differences between the sodium and bromide spaces, where it can be concluded that the distribution of bromide and sodium ions are probably on the whole similar.

The S^{35}-sulphate space in skeletal muscle is significantly lower than the Na^{24}-sodium space, probably due to the fact that the sulphate ions penetrate the muscle cells to a much smaller extent than sodium.

No difference was observed between the Na^{24}-sodium and S^{35}-sulphate spaces in the sinus and atrium ($0.5 > P > 0.1$), and neither was there any definite difference in the ventricle. It must be pointed out, however, that it is not easy to detect differences between distribution spaces for substances which are only present in the extracellular space, and those which though mainly extracellular enter the intracellular compartment to a very limited extent, especially if the ECV is large in relation to the intracellular volume, e. g. as in the sinus venosus and atrium.

The total sodium content

The values for the skeletal content of sodium in the heart and skeletal muscle determined with the aid of radioactive sodium conform well with the values found on analysis of stable sodium in toads kept for 6 months. In comparison with toads kept for 8 months, on the other hand, the value for skeletal muscle differs significantly ($P < 0.01$). This difference cannot at present be explained.

CHAPTER VII

ECV determinations and calculations of intracellular concentrations

This chapter discusses the values of the extracellular space obtained with different indicators and describes the calculations of the intracellular electrolyte concentrations. Factors of importance in penetration of ions and molecules into cells and through membranes are also discussed, together with the question of a Donnan distribution between the plasma and extracellular water.

Comparison of ECV determinations

In calculating the distribution volumes of the different substances, correction was made for the solute volume in the plasma (see chapter IV, page 40), but not for any possible Donnan effect between the plasma and the extracellular water.

The results of the space determinations are summarized in Figs. 15 and 16, and given more fully in Tables 6 (p. 42), 7 (p. 43), 9 (p. 50), 10 (p. 51), 11 (p. 54) and 12 (p. 60). The figures show the mean values for the different groups of determinations. In the figures there are three groups of columns for each tissue. The first groups represents the mean values of the halide spaces, determined on newly caught toads, and discussed in capter IV. It was shown there that there was no statistically significant difference ($P > 0.02$ in all cases) between the different halide spaces in any particular tissue.

In the second group the mean values for the C^{14}-mannitol, C^{14}-sucrose and S^{35}-sulphate spaces are combined. The investigations in this group were made mainly during the same period (the toads had been kept for 5 –7 months). The sulphate spaces were significantly greater ($P < 0.01$) than the cor-

responding mannitol spaces for all tissues. The sulphate spaces were also significantly greater than the corresponding sucrose spaces in the atrium and ventricle ($P < 0.01$) but not in the sinus and skeletal muscle ($P > 0.05$ and $0.05 > P > 0.02$ respectively).

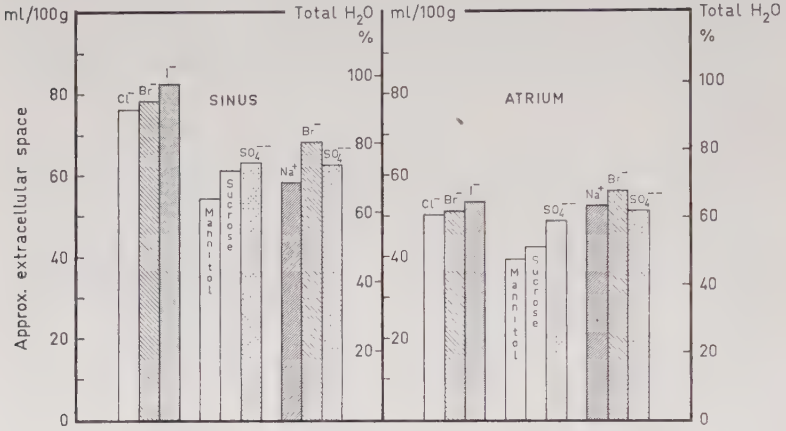

Figure 15. Mean values of space determinations made with different ions and molecules in the sinus and atrium. For further details, see text, page 63.
(Chloride spaces determined with stable isotopes, other spaces determined with radioactive isotopes or radioactive labelled substances.)

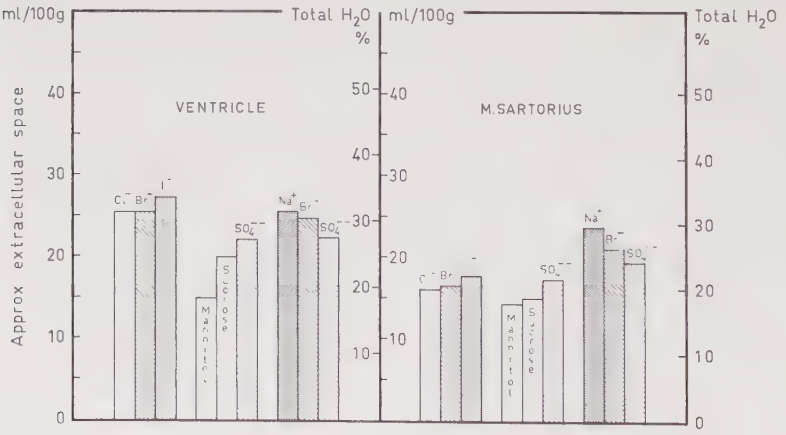

Figure 16. Mean values of space determinations made with different ions and molecules in the ventricle and sartorius muscle. For further details, see text, page 63.
(Chloride spaces determined with stable isotopes, other spaces determined with radioactive isotopes or radioactive labelled substances.)

The third group comprises simultaneous determinations of the Na^{24}-sodium, Br^{82}-bromide and S^{35}-sulphate spaces reported in Table 12 (p. 60). The Na^{24}-sodium and Br^{82}-bromide spaces were significantly greater than the C^{14}-mannitol and C^{14}-sucrose spaces in the atrium, ventricle and sartorius muscle ($P < 0.01$). The Br^{82}-bromide space differed significantly from the mannitol space ($P < 0.001$) in the sinus venosus but not from the sucrose space ($0.05 > P > 0.02$). No significant difference was observed between the Na^{24}-sodium space in the sinus and the C^{14}-sugar spaces ($P > 0.1$).

The Br^{82}-bromide space in the sinus venosus and sartorius muscle in the first group differed significantly from the corresponding space in the third group ($P < 0.01$) but not in the atrium and ventricle ($0.5 > P > 0.1$). This difference cannot at present be fully explained, but may be due to seasonal variations in the tissues.

The water content in the heart and skeletal muscle

In order to calculate the intracellular volume, the water content was determined according as described in chapter II, page 20. The results are given in Table 14. The water content is highest in the sinus venosus, followed by the atrium, ventricle and skeletal muscle in that order (significant difference between the different tissues, $P < 0.01$).

Table 14.

The water content in the heart and skeletal muscle in 22 toads. The table shows the mean values ± one standard deviation.

Preparation	Water content ml/100 g wet tissue
Sinus venosus	84.5 ± 1.1
Atrium	82.8 ± 1.6
Ventricle	81.2 ± 1.3
Sartorius muscle	80.0 ± 0.9

Intracellular sodium concentration

The intracellular concentrations of sodium and chloride were calculated from the experimental results, on which Table 12 is based. The following notation will be used:

ECV = the extracellular volume (ml/100 g wet tissue).
$[Na^{24}]_m$ = cpm Na^{24} per 100 g wet tissue.
$[Na^{24}]_p$ = Na^{24} concentration in plasma (cpm/l).
$[Na^{24}]_i$ = Na^{24} concentration in the intracellular water (cpm/l).
$[Na^{24}]_e$ = Na^{24} concentration in the extracellular water (cpm/l).
H_2O_m = water volume in the tissue (ml/100 g wet tissue).
$[Na]_p$ = sodium concentration in the plasma (meqv/l).
$[Na]_i$ = sodium concentration in the intracellular water (meqv/l).

The intracellular water volume (ICV) was calculated as the difference between the water volume and the ECV of the tissue, determined with different substances. The intracellular sodium concentration, $[Na]_i$, was calculated as the ratio $[Na^{24}] \cdot [Na]_p / [Na^{24}]_p$.

$[Na^{24}]_i$ was calculated from the determined molecular or ionic spaces according to the following formula:

$$[Na^{24}]_i = \frac{1000 \cdot [Na^{24}]_m - ECV \cdot [Na^{24}]_e}{H_2O_m - ECV}$$

where $[Na^{24}]_e$ was calculated in the same way as $[Br^{82}]_e$ as described in chapter IV. The results are given in Table 15.

The intracellular Na concentration determined on the basis of the sulphate spaces (determinations made simultaneously with the Na estimations) *did not differ between the different heart regions and skeletal muscle* (sinus-atrium $0.5 > P > 0.1$; atrium-ventricle $P \approx 0.5$; ventricle-skeletal muscle $0.05 > P > > 0.02$), and neither was there any significant difference when the mean values for the sulphate or sucrose spaces were used for the calculations (atrium-ventricle $P > 0.5$; ventricle-skeletal muscle $P = 0.1$). The mean value for $[Na]_i$ in the sinus was negative with these three methods of calculation (Table 15, lines 1, 2 and 4), which must depend on the method of calculation. The intracellular Na content is estimated as a difference between two almost equal numbers, which introduces a great uncertainty in the difference.

The intracellular Na concentration calculated by means of the mannitol spaces was higher in the atrium than in the

Table 15.

Intracellular concentrations of sodium [Na]i and chloride [Cl]i in the heart and skeletal muscle calculated with the aid of different ionic and molecular spaces. The table shows the mean values ± one standard deviation for the following number of determinations: sinus 8, atrium 9 and ventricle and sartorius muscle 10.

"Extracellular space"	[Na]i meqv/l				[Cl]i meqv/l			
	Sinus	Atrium	Ventricle	Sartorius muscle	Sinus	Atrium	Ventricle	Sartorius muscle
1. S^{35}-sulphate space (det. simultaneously with Na and Br)	− 5.5 ± 23.0	3.3 ± 14.8	6.3 ± 3.9	9.3 ± 3.2	24.1 ± 16.9	14.2 ± 10.3	5.1 ± 3.6	2.8 ± 2.7
2. S^{35}-sulphate space (mean value)	− 26.0 ± 15.3	9.1 ± 8.4	7.5 ± 6.7	11.9 ± 5.2				
3. C^{14}-mannitol space (mean value)	16.7 ± 12.4	33.9 ± 7.7	20.6 ± 6.6	18.3 ± 4.9	47.0 ± 17.1	34.3 ± 10.5	14.0 ± 3.0	9.9 ± 4.5
4. C^{14}-sucrose space (mean value)	− 15.8 ± 16.8	24.2 ± 8.2	19.6 ± 6.6	17.4 ± 5.0	33.0 ± 22.1	29.7 ± 11.4	13.2 ± 3.2	9.1 ± 4.5
5. Br^{82}-bromide space (mean value)	− 78.9 ± 24.6	− 27.9 ± 12.4	− 1.7 ± 8.2	5.8 ± 5.4				
6. Na^{24}-sodium space (mean value)					34.4 ± 22.4	7.7 ± 15.1	− 1.1 ± 3.9	− 4.5 ± 5.2

other tissues ($P < 0.01$). When the Br^{82}-bromide space was used as an approximation for the ECV, the $[Na]_i$ became negative in all regions of the heart. In the skeletal muscle, on the other hand, the $[Na]_i$ calculated from the bromide and sulphate spaces did not differ significantly ($0.1 > P > 0.05$) but was significantly lower than that based on the carbohydrate spaces ($P < 0.01$).

In the atrium, ventricle and striated muscle calculations based on the sulphate space gave a lower value of $[Na]_i$ *than when the carbohydrate spaces were used* ($P < 0.01$). No definite differences were, however, found for the sinus ($0.05 > > P > 0.02$); the variations for this tissue being rather large.

Intracellular chloride concentration

The intracellular Br^{82} concentration $[Br^{82}]_i$ was calculated on the basis of the different ionic or molecular spaces in the same way as $[Na^{24}]_i$. Assuming that Br^{82} is distributed similarly to the chloride ions, the intracellular chloride concentration $[Cl]_i$ was calculated as $[Br^{82}]_p \cdot [Cl]_i/[Br^{82}]_p$, where $[Cl]_p$ is a mean value from the analysis of plasma from 10 toads (see chapter IV). The results are given in Table 15.

The $[Cl]_i$, *calculated from the sulphate spaces*, is greater in the sinus than in the ventricle and skeletal muscle ($P < 0.01$), but is not significantly different from that of the atrium ($0.02 > P > 0.01$). The latter is higher than that of the sartorius muscle ($P < 0.01$) but is not significantly different from the sulphate based $[Cl]_i$ of the ventricle ($0.02 > P > 0.01$).

The *mannitol based* $[Cl]_i$ in the atrium and sinus is greater than that in the ventricle and skeletal muscle ($P < 0.01$). There are, however, neither significant difference between the $[Cl]_i$ values in the atrium and sinus ($0.1 > P > 0.05$) nor between the ventricle and skeletal muscle ($0.05 > P > 0.02$).

The *sucrose based* $[Cl]_i$ in the sinus does not differ significantly from that of the ventricle and atrium ($P > 0.5$ and $0.02 > P > 0.01$ respectively); the $[Cl]_i$ value of the atrium is, however, greater than that of the ventricle and skeletal muscle ($P < 0.01$).

The sulphate based $[Cl]_i$ *is less than that calculated from the carbohydrate spaces in the atrium, ventricle and skeletal muscle* ($P < 0.01$). There are no significant differences as regards the sinus.

Comparison between intracellular sodium and chloride concentrations

The intracellular sodium concentration is not significantly different from the chloride concentration in the atrium and ventricle ($P > 0.02$ in all cases) when calculated from the sulphate or carbohydrate spaces with simultaneous determinations of Na and Cl (Br). In the sinus the $[Na]_i$ calculated from the mannitol or sucrose spaces is lower than the $[Cl]_i$ ($P < 0.01$), but there is no significant difference when the calculations are based on the sulphate space ($0.05 > P > 0.02$). In the skeletal muscle the intracellular content of sodium is higher than the chloride content, regardless of which extracellular space indicator is used ($P < 0.01$).

Intracellular potassium concentration

The intracellular potassium concentration, $[K]_i$, was calculated from the extracellular space and the total potassium content (reported in chapter III, Table 3, toads kept in captivity for less than 3 months). The following notation will be used:

$[K]_m$ = meqv per kg wet tissue.
$[K]_p$ = potassium concentration in plasma (meqv/l).
$[K]_e$ = potassium concentration in the extracellular water (meqv/l).
$[K]_i$ = potassium concentration in the intracellular water (meqv/l).

$[K]_i$ is calculated according to the following formula:

$$[K]_i = \frac{[K]_m \cdot 100 - ECV \cdot [K]_e}{H_2O_m - ECV}$$

Table 16.

Intracellular concentration of potassium, $[K]_i$, in the heart and skeletal muscle calculated with the aid of different ionic and molecular spaces and the total content of potassium (reported in Tables 2 and 3, chapter III). The table shows the mean values ± one standard deviation for the following number of potassium determinations: sinus, atrium and ventricle 14, and skeletal muscle 20.

"Extracellular space"	$[K]_i$ meqv/l			
	Sinus	Atrium	Ventricle	Sartorius muscle
1. S^{35}-sulphate space	104 ± 33	101 ± 21	99 ± 11	114 ± 8
2. C^{14}-mannitol space	75 ± 20	79 ± 15	89 ± 9	109 ± 7
3. C^{14}-sucrose space	97 ± 33	85 ± 15	90 ± 10	110 ± 8
4. Na^{24}-sodium space	87 ± 14	112 ± 23	105 ± 12	127 ± 9
5. Br^{82}-bromide space	138 ± 34	128 ± 35	104 ± 11	121 ± 9

where $[K]_e$ is calculated in the same way as $[Br^{82}]_e$ (see chapter IV). The results of the calculations are given in Table 16. The intracellular potassium concentration $[K]_i$ calculated on the basis of the mannitol, sucrose, sulphate or sodium spaces is lower in the atrium and ventricle than in the sartorius muscle, and the ventricle is also lower than the sartorius when the bromide space is used ($P < 0.01$). The intracellular potassium concentration in the sinus is lower than in the skeletal muscle when calculated from the mannitol or sodium spaces ($P < 0.01$), but not when calculated from the other spaces ($P > 0.05$).

The $[K]_i$ *showed no differences in comparisons between different heart regions, regardless of which substance was used for the ECV determinations* ($P > 0.1$ in most cases, but $P > > 0.02$ in all cases), except that with the calculations based on the sodium space the $[K]_i$ in the sinus was lower than that of the atrium and ventricle ($P < 0.01$).

The mannitol-based $[K]_i$ is lower than the sodium and bromide $[K]_i$ values in the ventricle and skeletal muscle, and is also lower than the potassium concentrations calculated from the sulphate spaces in the atrium and ventricle ($P < 0.01$). There are no differences for the sucrose-based $[K]_i$ values in any of the tissues ($P > 0.5$).

The sinus $[K]_i$ values calculated from the mannitol or sucrose spaces are lower than that based on the bromide space ($P < 0.01$), but do not differ from the values calculated from the other spaces ($P > 0.1$).

On calculating the intracellular potassium concentration, e. g. from the sulphate space, in toads which had been kept in captivity for a long period (not reported in the table), it was found that the $[K]_i$ was lower in the atrium, ventricle and skeletal muscle ($P < 0.01$) but that there was no definite decrease in the sinus ($0.05 > P > 0.02$).

DISCUSSION

Comparison of ECV between different tissues studied

It is apparent that the sinus venosus has the greatest ECV, followed by the atrium, ventricle and skeletal muscle, *regardless of which substance is used for the ECV determinations* (there was no significant difference, however, between the mannitol and sucrose spaces in the ventricle and the corresponding spaces in the skeletal muscle; $P > 0.02$).

Relationship between the histological findings and the calculated values of the spaces

The extracellular volume in the muscle, as assessed from the relative surface area of the space between the fibres in histological sections, corresponds approximately to the chloride space, and this was one of the reasons that the chloride space became used as an approximation for the ECV (Fenn, 1936). Sandow (1939) calculated theoretically the space between tightly packed parallel cylinders with the same fibre diameter as the muscle fibres in the frog sartorius muscle, as 15.4 % of the entire muscle volume. This value was in good agreement with experimental values for the chloride space.

No similar comparisons between histological and physiological findings have been made in the heart. From histological investigations on the frog heart (Kisch, 1961) and from data about the conduction system in mammals (see Kawamura, 1961, among others), the conclusion may be drawn

that the ECV is large both in the sinus venosus and in the conduction system, and is to a great part occupied by collagen fibres.

It is possible that *connective tissue* and *fatty tissue* constitute a source of error in determinations of the ECV, since it is not certain that the test substances penetrate these tissues to the same extent as other parts of the ECV. The neutral fat content in the tissues studied was about 1 % of the weight of the wet tissue (see Table 1, chapter II), and has therefore been considered unimportant. The connective tissue content has not been investigated, but may possibly be of some significance, at least in determinations of the ECV in the sinus venosus, since it is known that this tissue is rich in connective tissue (KISCH, 1961).

One possible explanation for the fact that the distribution spaces for the carbohydrate molecules are smaller than the corresponding ionic spaces is, according to SELKURT (1963) that it is only with difficulty that mannitol and sucrose penetrate the connective tissue, which should mean that these spaces are smaller than the actual extracellular volume. If the matrix of collagen fibres which pervades the extracellular space contains high polymers such as hyaluronic acid or other macromolecules it is possible that some steric exclusion may occur if large test molecules are used to indicate the ECV. Thus inulin (average mol wt $\approx$ 5000) may not have access to as large an extracellular space as a smaller molecule such as mannitol (OGSTON and SHERMAN, 1961) (see also discussion, p. 79).

It is evident that the absolute values for the intracellular concentrations are strongly dependent on the size of the extracellular volume, i. e. it is of great importance to use the measurement for the space which is most representative for the physiological extracellular space (cf. formulae on pages 66 and 69).

In determinations of the ECV in the heart, the sulphate space has been assessed as the most correct approximation, since its distribution volume rapidly equilibrates, after which the volume does not appear to increase even after a long period. It has further been considered by other authors (see

PAGE, 1962, *inter alia*) that this ion remains essentially extracellular. PAGE (1962) showed that the volume of the heart muscle cells did not increase, but remained constant, when the K concentration was raised isosmotically—by exchange of the Na ions—in the bath solution, the only anion of which consisted of impermeable sulphate ions. When corresponding experiments were made with the permeable chloride ion, the intracellular volume increased greatly. PAGE interpreted these experiments as indicating that the sulphate ions remained extracellular.

It is probably less suitable to use the mannitol space, for example, as an approximation for the ECV, since it is claimed that mannitol penetrates the connective tissue only very slowly (SELKURT, 1963), and since the distribution volume of mannitol becomes very large after a long period (see chapter V), which probably indicates permeation into the cells.

The water content

The water content is highest in the sinus venosus, followed by the atrium, ventricle and skeletal muscle in that order. It is remarkable how little the water content varies among different preparations.

Factors of significance in the permeation of solutes into cells

The transport of molecules or ions into living cells or through membranes is determined by several factors, such as their concentration, mobility and driving forces (TEORELL, 1953). Several factors influence the permeability of a membrane to a molecule or ion, namely the properties of the molecules or ions themselves (e. g. size, hydration, solubility, charge) and properties of the membrane (pore size, physico-chemical structure, charge, affinity for solvents etc.). The driving force may be a concentration gradient, electrical potential gradient or a hydrostatic pressure gradient (TEORELL, 1953). The electrical potential gradient does not influence directly undissociated or uncharged species.

Properties of the molecules or the ions themselves

The properties which influence the permeability to molecules include the size, hydration and solubility in the membrane. Of the carbohydrate molecules used, the calculated molecular model radius of mannitol is 4.5 Å. (For a comparison of the radii of small molecules determined in different ways, see SCHULTZ and SOLOMON, 1961.) (Maltotriose is certainly considerably greater, but may break down in the tissue.) The carbohydrate molecules used may be able to penetrate the membrane in several principally different ways, i. e. by means of pre-formed pores, by solution in the membrane or by other chemico-metabolic processes. Of the molecules used, mannitol and sucrose pass through the endothelial membrane between the heart lumen and the extracellular space with ease, while penetration of the cell membrane takes place considerably more slowly (PAGE, 1962).

PAGE (1962) found that sucrose and mannitol (molecular model radii 4.5 Å and 4.0 Å respectively, see SCHULTZ and SOLOMON, 1961) were the smallest molecules which appeared to remain extracellular, at least in the heart muscle in the cat. Experiments reported here show that the mannitol and sucrose spaces became very large after long periods of distribution, at least in the atrium and ventricle, which would seem to indicate some penetration of the cell membrane.

The sizes of the ions used for determinations of approximate extracellular spaces (ionic spaces) are shown in Table 17.

Table 17.

The crystal and hydrated radii of different ions, calculated from the Stokes-Einstein equation. r_x = crystal ion radius, r_H = hydrated radius. Data obtained from NIGHTINGALE (1959).

Ion	r_x Å	r_H Å
Na^+	0.95	3.58
K^+	1.33	3.31
Cl^-	1.81	3.32
Br^-	1.95	3.30
I^-	2.16	3.31
SO_4^{--}	2.90	3.79

where both the crystal and the so called hydrated radii are given. These radii are calculated from the behaviour of ions in solution and are somewhat controversial (see SAMOILOV 1957). The hydrated radii of the different halide ions are very similar, but somewhat smaller than the corresponding radii of the sodium and sulphate ions. The calculated sizes of the ions and molecules used are compared in Fig. 17.

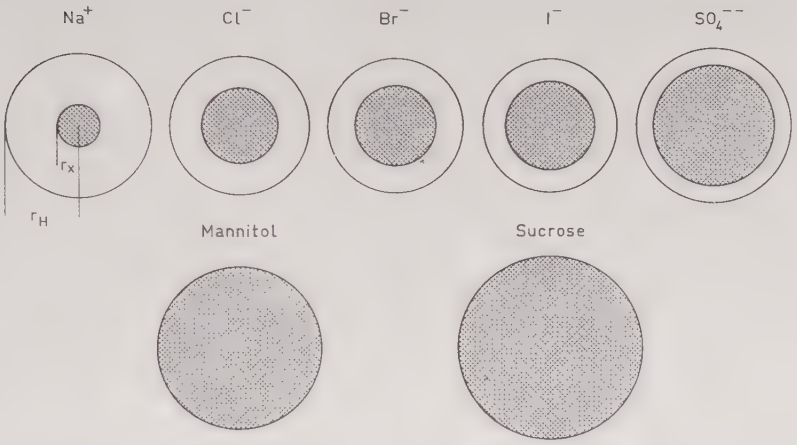

Figure 17. The sizes of the ions and molecules used for the ECV determinations. r_x = crystal radius. r_H = hydrated radius.

Properties of the membrane

The extent to which the different ions penetrate the cell membrane depends not only on the porosity (i. e. some function of the structure and size of the penetrant) but also on the membrane charge. There are reasons for believing that the heart cell membrane is negatively charged, the negative ions thus being retarded (see TEORELL, 1953).

Driving forces

For both ions and molecules, the chemical potential gradient as also any presence of a hydrostatic pressure gradient influences the distribution between the extra- and intracellular

spaces. For ions, in addition, there is the influence of the electrical potential gradient. Fore more detailed studies of the permeability of natural membranes and of the laws which determine the distribution of ions and molecules in biological systems, reference may be made to DAVSON and DANIELLI (1952), TEORELL (1953) and HARRIS (1960), *inter alia*.

The importance of the distribution period

As shown by the results reported in chapter V, it is seen that the sulphate spaces very rapidly reach their maximal values, while the mannitol and sucrose spaces continue to increase for long periods, at least in the atrium and ventricle, and exceed the corresponding halide spaces. This may be interpreted as indicating that the sulphate ion remains extracellular or if it does penetrate the cell it does so to only a very small extent, while the carbohydrate molecules slowly enter the cell.

Other ions used (sodium and halide ions) equilibrate rapidly with the stable isotopes (see chapter IX and also MANERY and BALE, 1941).

The importance of the Donnan factor

The extracellular fluid is assumed to be an ultrafiltrate of plasma, the electrolyte composition of which can be calculated if correction is made for the water content of the plasma and for the Donnan equilibrium which is assumed to prevail between the plasma and the extracellular fluid.

The theoretical Donnan factor for the distribution of univalent negative ions between plasma and protein-free ultrafiltrates in man has been calculated as approximately 0.96. For the chloride and sodium ions there was good agreement between the predicted and observed values (VAN SLYKE, WU and MCLEAN, 1923). MANERY (1954) compared values for the Donnan factor (r) from the literature (obtained by ultrafiltration *in vitro* or from simultaneous electrolyte determinations in plasma and oedema fluid). This author considered that the factor $r = 0.95$ should be used for all negative ions.

For positive ions, e. g. Na and K, the Donnan factor is somewhat greater than unity, while the correction factor for plasma water is somewhat less than unity, and these factors therefore tend to cancel each other out (see chapter IV, page 41), whereby the concentration of positive univalent ions in the extracellular water may be assumed to be the same as in the plasma (cf. BERGSTRÖM, 1962).

For negative ions both the Donnan and the correction factor for the plasma water act in the same direction, which means that the concentrations of negative ions are higher in the extracellular water than in the plasma. For the divalent sulphate ion the Donnan factor ($\sqrt{r_{SO_4}}$) should theoretically be r_{Cl}^2, i. e. of the order of $0.96^2 = 0.92$ if the sulphate ion were the predominant diffusible anion and if conditions were otherwise the same (DAVSON, 1956).

For comparison it may be mentioned that SWAN, FEINSTEIN and MADISSO (1956) found that the equilibration concentration of radioactive sulphate in serum was 0.95 that of the sulphate concentration in a dialysate of human serum, and that the corresponding value for dog serum was 0.96. The corresponding factors for chloride were 0.99 and 0.98 respectively. Cellophane membrane was used for dialysis (this is weakly negatively charged). These experiments showed that the Donnan factor for the sulphate ions is $\approx r_{Cl}^2$.

Of decisive importance to the ion distribution between the plasma and extracellular water is the construction and charge of the membrane which separates the lumen from the extracellular space. This consist of a single endothelial layer under which there is a layer of collagenous connective tissue. As mentioned previously (chapter II), the toad heart lacks coronary vessels.

In the calculations made here no correction has been made, however, for any Donnan distribution of ions between the plasma and the extracellular water, since the protein content in plasma is so low and there are considerably practical and theoretical difficulties in determining a correct value for the Donnan factor for the ions used. In order to make an accurate correction for the Donnan effect, the properties of the membrane, which divides the plasma from the extracellular space

should be known, as also other factors of importance in ion distribution, such as the type of plasma proteins, the concentration and isoelectric points, and the plasma pH, etc. The correction is probably of little importance to the calculations. (There is no significant difference between intracellular concentrations calculated with or without the Donnan factor = = 0.96; $P > 0.05$ in all cases). Correction has only been made, therefore, for the solid plasma content (see chapter IV, page 40).

Intracellular electrolyte concentrations

The intracellular concentrations of Na and K did not differ between different regions of the heart, and in the case of Na neither was there any difference between the heart and the skeletal muscle, while the intracellular potassium content was lower in the heart than in the sartorius muscle. The intracellular chloride concentration in the sinus and atrium was greater than the $[Cl]_i$ in the sartorius, while the intracellular content in the ventricle did not differ from that in the sartorius.

In the heart the $[Cl]_i$ in the sinus was greater than that in the ventricle, but did not differ significantly from the intracellular chloride content in the atrium. These differences in calculated intracellular concentrations may depend either on true intracellular differences or on differences between different parts of the ECV, e. g. in the connective tissue, which are not completely penetrated by the substance used for the ECV determination. In calculating the results the chloride in a site such as the connective tissue, which is not penetrated by the ECV indicator, is assigned an intracellular location. The latter explanation does not seem plausible, however, because the intracellular Na content, determined simultaneously with the $[Cl]_i$, was very low when calculated from the sulphate or mannitol spaces, and it is therefore probable that the entire or almost entire ECV was measured.

The intracellular contents of Na and Cl are lower when calculated from the sulphate spaces than when calculated from the carbohydrate spaces, while $[K]_i$ is lower when based on the mannitol space than when calculated from the sulphate spaces

in the atrium and ventricle (no significant differences in the sinus or sartorius muscle). HAJDU (1953) found in the frog ventricle that the ratios of extra- to intracellular concentrations of Na and K were approximately 6 : 1 and 1 : 36 respectively (calculated from the inulin space), which should correspond to an intracellular Na concentration of about 20 meq/l water and a K concentration of about 110 meq/l water. The sodium value is in good agreement with the ventricular $[Na]_i$ calculated from the mannitol space, but is higher than the value obtained from calculations from the sulphate spaces, probably because the inulin spaces are usually smaller than spaces determined with smaller carbohydrate molecules (see PAGE, 1962) or ions (MANERY, 1954). The value for $[K]_i$ calculated according to HAJDU's values conforms well with those found here. This is no doubt partly due to the fact that the ECV value is of less importance in calculating $[K]_i$ than $[Na]_i$.

LAMB (1961) found that the $[Cl]_i$ in the rabbit atrium was 19.5 meq/l water, and PAGE (1962) found an intracellular chloride concentration in the cat heart muscle of 17 meqv/l, which values are of the same order of size as those found here in the sinus and atrium, calculated from the sulphate spaces. The intracellular concentrations of Na and Cl in the skeletal muscle of the frog are in good agreement with values reported by HARRIS (1960), while the $[K]_i$ is lower than the values found by HARRIS, which may be due to differences between toads and frogs, or the condition of the animals, etc.

The difference in the total electrolyte contents in different regions of the heart can thus, as regards Na and K, be explained by differences in the extracellular volumes, while the variations in the chloride content appear to be due partly to differences in the extracellular volume and partly to differences in the intracellular chloride concentration.

PART II

CHAPTER VIII

Introduction

As mentioned in the introduction to part I, much attention has been paid in recent years to attempts at explaining how the asymmetry of ions between the intra- and extracellular phases occurs and can be maintained, and also how excitability arises. BOYLE and CONWAY (1941) put forward a hypothesis regarding the properties of the cell membranes to explain the ionic asymmetry and the resting potential in striated muscle. The diffusion and active transport of substances through the cell membrane have been studied extensively by USSING (1949, 1951). According to the theory of HODGKIN and HUXLEY (1952 *a–d*) the ion distribution at rest is maintained by an active outward transport of sodium ions from the cells, combined with a low sodium permeability. This theory, based mainly on results from squid axons, explains the resting potential as a result of the difference in concentration between the intra- and extracellular spaces.

According to TEORELL (1960), the electrochemical potential gradient together with a hydrostatic pressure gradient may give rise to a flow of water which in its turn should result in a separation of ions in the charged pores in the membrane. According to this theory the water transport between the cellular and extracellular phase could play a large part in the occurrence of the electrical phenomena and ion dyssymmetry (TEORELL, 1962).

In order to explain the depolarization of the cell membrane during an action cycle in an excitable cell, it was assumed that the permeability of the cell membrane was changed so that the sodium permeability became much greater than the potassium permeability (HODGKIN and HUXLEY, 1952 *a–d*).

NOBLE (1960, 1962) developed a theory which based on the

HODGKIN and HUXLEY theory and which took into consideration the special physiological conditions in the heart tissues. A theoretical discussion on the sodium and potassium hypothesis has been presented by BRADY and WOODBURY (1960).

Numerous studies have been reported (see e. g. BROOKS, HOFFMAN and ORIAS, 1955; WEIDMANN, 1956 and HOFFMAN and CRANEFIELD, 1960) which support the assumption of a relationship between the electrical activity and ionic flow in excitable tissue. Attempts have been made, further, with different models of oscillatory or instable systems, to reproduce the electrical phenomena which occur in excitable tissues, such as resting and action potentials, the tissue's own rhythmicity etc. Common to all these theories is the importance of ion transport. By alteration of forces influencing the system or the membrane attempts have been made to produce rhythmic changes in the system in order to simulate the natural conditions in excitable tissues, e. g. the effect of acetylcholine.

It has long been known that acetylcholine and vagal stimulation influence the ionic transport in the heart. HOWELL and DUKE (1908) showed that vagal stimulation liberated potassium ions into the perfusion solution which flowed through the heart. DALE (1937) showed that LOEWI's "Vagusstoff" (1921) was the substance which was released on vagal stimulation and which produced changes in the heart muscle. Since this time the mode of activity of the acetylcholine released on vagal stimulation has presented a great problem. It has long been known that the potassium ions are affected on vagal stimulation (LEHNARTZ, 1936; HOLLAND, DUNN and GREIG, 1952 *a*, *b*). FATT and KATZ (1951) put forward the hypothesis that the transmitter substance gave rise to a non-specific increase of the ionic permeability of the cell membrane. BURGEN and TERROUX (1953) presented experimental results which showed that acetylcholine acted on the heart muscle by changing the permeability to potassium ions, which was confirmed by SEKUL and HOLLAND (1959), HARRIS and HUTTER (1956) and HUTTER (1961), among others. Some investigations (ÖBRINK and ESSEX, 1953 and KLEIN and HOLLAND, 1958) indicate that the acetylcholine or vagal stimulation influences the heart cell membrane's permeability not only to potassium

ions but also to sodium ions. The concept now generally prevailing is, however, that the acetylcholine or vagal stimulation give rise to a selective increase in permeability to potassium ions in the heart, since few investigations support the view that the transport of other ions also is influenced by acetylcholine.

The aim of this part of the investigations was *to study the electrolyte distribution in the vagus-stimulated heart, and to study the exchange of ions between the heart and blood on vagal stimulation in order to seek a basis for chronotropic changes.* In the experiments the distribution of sodium, potassium and chloride (bromide) in the vagus-stimulated heart is compared with the distribution under normal conditions.

CHAPTER IX

The sodium, potassium, bromide and sulphate spaces in the heart on vagal stimulation

As mentioned in the introduction to part II acetylcholine is considered to act on the heart by inducing an increase in the potassium permeability of the cells. Whether or not the transport of other ions, e. g. sodium and chloride ions, is also influenced by acetylcholine and vagal stimulation is somewhat problematical.

The importance of chloride and other ions in the formation and propagation of impulses in excitable tissue is still relatively unclear. It has long been considered that the anions are passively distributed (as a result of the distribution of the sodium and potassium ions; HODGKIN & HUXLEY, 1952 *a–d*) and thus do not contribute to any noteworthy extent to the resting potential. TASAKI (1963) showed on intracellularly perfused squid axons that the permeability to a univalent anion, Br^{82}, was very small. TASAKI and SPYROPOULOS (1961) showed that stimulation of the axon did not result in an increased flow of Br^{82} or other anions. Complete exchange of intra- and/or extracellular chloride for sulphate or glutamate changed neither the resting nor the action potential in squid axons to any significant extent (TASAKI and SHIMAMURA, 1962). These findings support the concept that the membrane of the axon has fixed negative charges, which was postulated already in 1936 by TEORELL.

CARMELIET (1961) found in Purkinje fibres that the chloride ions contributed very little to the resting potential, but by resistance measurements he found that during the action potential the chloride ions contributed to repolarization by migrating into the cells.

In other words it is uncertain whether there is any flow of anions, especially chloride ions, through the cell membrane associated with the electrical activity in excitable tissue. It is also unknown whether the transport of anions through the cell membrane is influenced by acetylcholine. SEKUL and HOLLAND (1959) did not find an effect of acetylcholine on the fluxes of Ca^{45} and Cl^{36} in isolated rabbit atria. Neither did HUTTER (1961) observe any influence of acetylcholine or vagal stimulation on the transport of bromide ions in isolated sinus venosus.

The ventricular muscle and Purkinje fibres have been reported to be very insensitive to acetylcholine and vagal stimulation (HOFFMAN and SUCKLING, 1953; HOFFMANN and CRANEFIELD, 1960) since only very high concentrations of acetylcholine influence the action potential (HOFFMANN and SUCKLING, 1953). Certain authors consider that this insensitivity to acetylcholine only holds for mammalian ventricle, and not for that of amphibians, but that the effect on the ventricle in frogs, for example, is smaller than on the atrium and sinus venosus. Thus in the frog ventricle acetylcholine increases the permeability to potassium ions (HOLLAND, DUNN and GREIG, 1952 *a*) and hastens repolarization (LÜCKEN and SCHÜTZ, 1938).

The aim of this part of the investigation was *to study (a) the transport of radioactive sodium, potassium and bromide ions on the normal and vagus-stimulated heart, (b) the extracellular volume (the sulphate spaces) on vagal stimulation and (c) the total sodium and potassium content in the normal and vagus-stimulated heart and to compare any effects of vagal stimulation in different regions of the heart.*

GENERAL PRINCIPLES

When a radioactive isotope is injected into the vascular system of an organism the isotope distributes rapidly in the plasma. After a short period (a few minutes) the extracellular space reaches almost the same specific activity as the plasma, while the intracellular specific activity increases consdierably more slowly. This means that after short distribution periods (< 20 minutes) it is extremely unlikely that the specific activity is everywhere the same. After long distribution periods (> 10

hours) such radioactive isotopes as Na^{24}, Br^{82} and K^{42} can be regarded as completely or almost completely equilibrated with stable, easily exchangeable isotopes.

If the heart is influenced *after a short distribution period*, e. g. by vagal stimulation, and this induces a different radioactive isotope content in the heart compared with the normal heart, this effect may be explained in two ways. Firstly it may be due to an alteration in the exchange of the radioactive isotope between the heart and the blood without any change in the total electrolyte content. Secondly the effect may take place by means of an *altered total content of the electrolyte*, i. e. vagal stimulation would in this case cause a *net flow* of the electrolyte to or from the heart.

After a long distribution period, when the radioactive isotopes can be considered to be completely or almost completely equilibrated with stable isotopes, the relative radioactive isotope content is a measure of the stable electrolyte content. If vagal stimulation now results in a change in the radioactive isotope content in the heart, this indicates a shift in the total electrolyte content in the heart, i. e. in this case vagal stimulation has caused a net flow of the electrolyte to or from the heart.

METHODS

Toads were used for all the experiments (either *Bufo bufo*, newly caught or kept for about 7 months, or, for the series with bromide ions, newly caught *Bufo viridis*).

Scheme for the studies of the influx of ions into the heart on vagal stimulation

In order to study the effect of vagal stimulation on the influx of the radioactive ions into the heart, the following scheme was used. Two series of experiments were made, one on normal toads (the control group) and the other on vagus-stimulated animals. The radioactive isotope was injected in all cases and was allowed to distribute for periods of varying lengths. The toad was then pithed and the heart dissected free. In a number of animals blood samples were taken from the

left aorta. The sinus venosus, atrium and ventricle were then excised, together with a sample from the skeletal muscle (sartorius). The content of radioactive isotopes in the tissue samples was then determined.

Vagal stimulation

In some of the toads, in addition to the usual heart dissection, one of the vagus nerves was dissected free approximately 1–1.5 cm from the heart—as a rulè the right vagus nerve, since this is somewhat larger than the left. An electronic stimulator (Kistner lab stimulator, Stockholm, Sweden; square pulse. 15 volts, frequency 20 per second, duration 5 msec) and silver electrodes (distance between the electrodes about 2 mm) were used in the experiments. YOUNG and UPHAM (1961) found that the longer the pulse duration used the longer was the cardiac arrest obtained. The longest period of cardiac arrest was obtained with a stimulation frequency of 15–60 pulses per sec and a pulse duration of 6 msec.

When the radioactive isotope had been allowed to distribute for the intended time the vagus nerve was stimulated, and when cardiac arrest and subsequent vagal escape had been observed the heart was excised as quickly as possible. The stimulation of the vagus nerve was continued while the tissue samples were being taken, to avoid the possible disappearance of any effects of the stimulation on the distribution and transport of the ions in question. The total number of stimulation pulses was 200–600. The procedure was otherwise the same as for the control series.

Reference for the relative radioisotope concentration

In order to compare the results from different toads, the ratio between the concentration of the radioactive isotope in the heart and the concentration in the sartorius muscle or in the plasma was determined, i. e. muscle or plasma was used as a reference to express the *relative content* of the radioactive isotopes in the different regions of the heart.

With short distribution periods (< 1 hour) plasma seemed to be a better reference than skeletal muscle, since the sartorius

muscle sometimes exhibits large variations in isotope concentration among different toads after the same short distribution period. DANIELSON (1962), for example, concluded that vagal stimulation gave rise to a higher equilibration rate for Na^{24} since vagus-stimulated hearts had higher Na^{24} contents relative to skeletal muscle than normal hearts after the same short distribution period. In these experiments it was probably the skeletal muscle reference which varied and which had a lower Na^{24} content in vagal-stimulated than in normal animals (probably due to bad circulation in the vagus-stimulated toads because longer handling time after pithing and before injection of the radioisotopes). The ratio between the radioactive isotope concentrations in the heart and plasma varied considerably less than the ratio between the concentrations in heart and skeletal muscle (in comparisons between different toads) even with short distribution periods, and this reference was therefore used in most cases.

Experiments with Br^{82}

Approximately 1 ml normal Ringer solution containing 10–15 μC Br^{82} (74 mC/g Br) were injected into the posterior lymph sac of each of 40 toads. After various distribution periods (5 mins–19 hrs) the toads were pithed and the heart dissected free. The sinus venosus, atrium, ventricle and a piece from the left sartorius muscle were then excised, blotted gently on filter paper, weighed and analysed for their contents of Br^{82} (as described in chapter II, page 22). Corresponding experiments were made with vagal stimulation of the heart (as described above).

Experiments with K^{42}

Approximately 1 ml radioactive Ringer solution containing 25 μC K^{42} was injected into the posterior lymph sacs of a number of newly caught toads. The isotopes were allowed to distribute for 10–32 hours, after which the toads were pithed and the heart dissected free. Heart and skeletal muscle samples were then taken—in part of the toads after vagal stimulation—

and weighed and analysed for their contents of K^{42} (see chapter II, page 22).

The Na^{22} or Na^{24}-sodium, K^{42}-potassium, Br^{82}-bromide and S^{35}-sulphate spaces in the heart and skeletal muscle

Approximately 1 ml radioactive Ringer solution containing 5 μC Na^{22} (spec. act. 2 mC/g Na) *or* a mixture of 15 μC Na^{24} (spec. act. 6 mC/g Na), 15 μC Br^{82} and 5 μC S^{35} (carrier free) *or* a mixture of 15 μC K^{42} (spec. act. 1.5 mC/g) and 5 μC S^{35} was injected into the posterior lymph sac of each toad (5, 6 and 10 toads were used for the three different isotope solutions respectively), the isotopes were allowed to distribute for periods of different lengths (5–55 minutes), the toads were pithed, and blood and tissue samples were taken and weighed (as described in chapter II, page 13). Duplicate plasma samples and the other tissue preparations were then placed on combustion papers (for later analysis of S^{35}) and dried, after which the content of the gamma-radiating isotopes was determined (see chapter II, page 21). In the determination of Na^{22} direct analysis was made without previous drying.

When the blood samples had been collected, the vagus nerve in some of the toads was stimulated, after which the procedure was the same as for the normal toads.

When at least 10 half lives for Br^{82} had elapsed, the samples were burned and analysed for their content of S^{35} by liquid scintillation counting (see chapter II, page 23).

The Na and K contents in the heart and skeletal muscle

In a series of experiments the total content of sodium and potassium in different regions of the heart and skeletal muscle in normal toads was compared with that in the corresponding tissues of vagus-stimulated toads. These experiments were identical with those described above for K^{42}, with the exception that no Ringer solution was injected. Since the quantity of sodium and potassium in the sinus venosus and atrium is small, samples from several toads were pooled and burned together (as described in chapter II, page 15), after which the mean values for the combined samples were determined.

RESULTS

The effect of vagal stimulation on the transport of Br^{82} in the heart

Figures 18–20 show the relative Br^{82} concentration in different heart regions (*in relation to the skeletal muscle*) in normal and vagus-stimulated toads (*Bufo viridis*) after different distribution periods (1–20 hours). The ratios for the different heart regions are all > 1, i. e. the concentration of Br^{82} in the heart is larger than in the skeletal muscle. The greatest ratio is that between the sinus venosus and skeletal muscle, followed the atrium and ventricle, (cf. chapter IV). The ratios for all the heart regions show a relatively wide range. There are no significant differences for the ratios in any region of the heart between *normal* and *vagus-stimulated* toads, either after short or long distribution periods for Br^{82}.

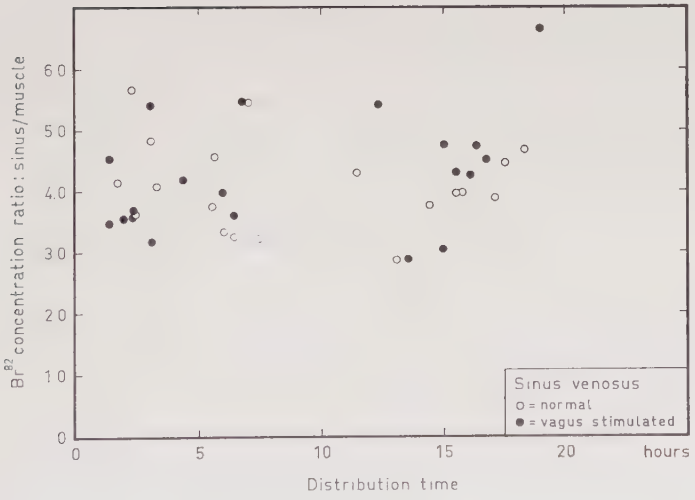

Figure 18. The relative Br^{82} concentration in the sinus venosus (sinus/sartorius muscle) in normal and vagus-stimulated toads (*Bufo viridis*).

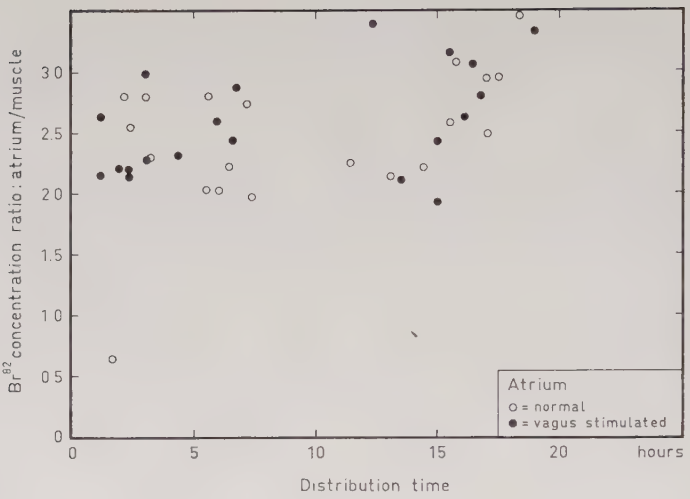

Figure 19. The relative Br^{82} concentration in the atrium (atrium/sartorius muscle) in normal and vagus-stimulated toads (*Bufo viridis*).

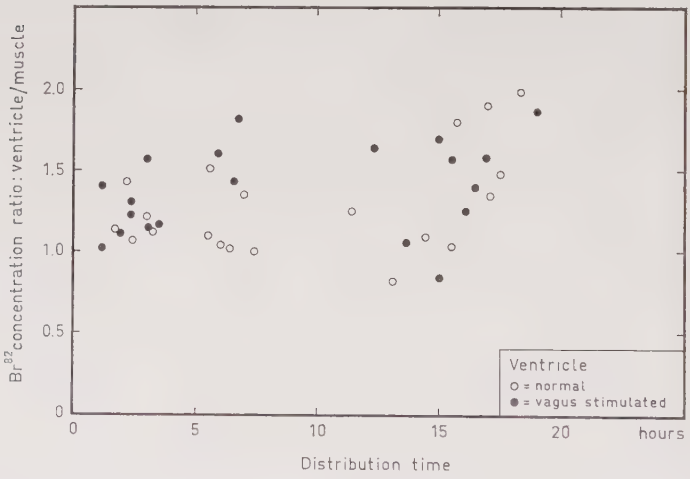

Figure 20. The relative Br^{82} concentration in the ventricle (ventricle/sartorius muscle) in normal and vagus-stimulated toads (*Bufo viridis*).

The Br^{82}-bromide, Na^{22}- or Na^{24}-sodium and K^{42}-potassium spaces in the heart and skeletal muscle

Figures 21 and 22 show the Br^{82}-bromide spaces (i. e. the Br^{82} concentration in the heart relative to the Br^{82} content in

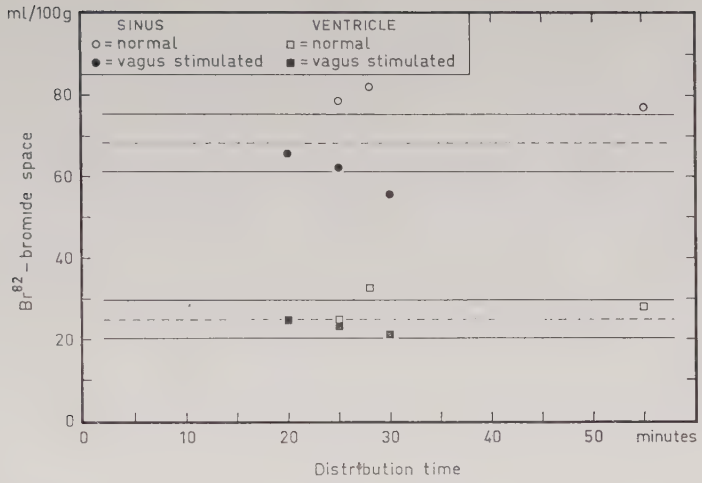

Figure 21. The Br^{82}-bromide spaces in the sinus venosus and ventricle in normal and vagus-stimulated toads after short distribution periods. The lines show the mean value ± 2 S.D. for the Br^{82}-bromide spaces after distribution periods of 22—50 hours (see chapter VI).

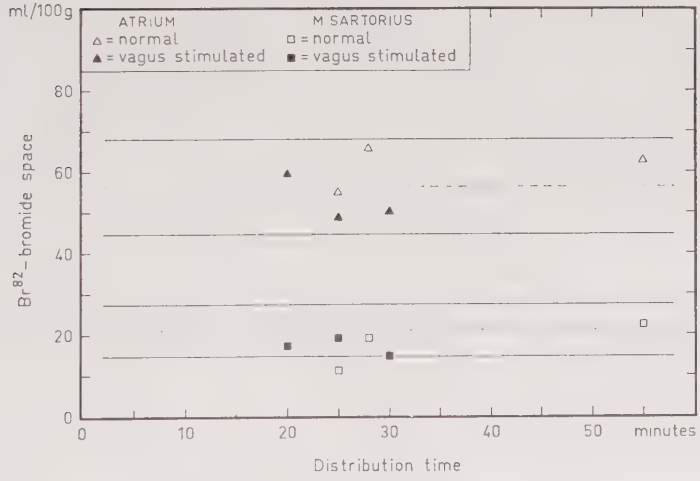

Figure 22. The Br^{82}-bromide spaces in the atrium and sartorius muscle in normal and vagus-stimulated toads after short distribution periods. The lines show the mean value ± 2 S.D. for the Br^{82}-bromide spaces after distribution periods of 22—50 hours (see chapter VI).

plasma), and figures 23 and 24 show the Na^{22}- or Na^{24}-sodium spaces in the heart and skeletal muscle in normal and vagus-stimulated toads after short distribution periods. The Na^{22}- or Na^{24} has not had time to equilibrate completely with the stable sodium in the skeletal muscle (Fig. 24), which is evident by the fact that with short distribution periods the sodium space (determined from the Na^{22} or Na^{24}) is considerably smaller than the values found after a distribution period of about 22–50 hours. For the heart the Na spaces after a short distribution period lie well within the range obtained when radioactive sodium had been allowed to distribute for 22 hours or longer (see chapter VI). Among the reasons for the heart more rapidly reaching the maximal values for the sodium space are probably a better nutrition and a relatively larger extracellular volume.

If one part of the heart, e. g. the atrium, is compared in control and vagus-stimulated animals there is no significant difference as regards the radioactive bromide- or sodium spaces. Figure 25 shows a comparison between the mean values of the Br^{82} or Na^{22}-Na^{24} spaces for normal and vagus-stimulated hearts (expressed as percentages of the mean value of the corresponding Br^{82}-bromide spaces or Na^{24}-sodium spaces found with distribution periods longer than 22 hours, see chapter VI). Both for the bromide and sodium ions, the mean values appear to be smaller for vagus-stimulated than for normal hearts. Assuming that vagal stimulation has the same effect on all regions of the heart, the results for the entire heart can be combined into one single large sample (i. e. one for Na and one for Br). *A comparison between normal and vagus-stimulated hearts in this combined material shows that the Br^{82} spaces in vagus-stimulated hearts are significantly smaller than in normal hearts ($P < 0.01$), while for the Na^{22}- or Na^{24}-sodium spaces the difference was not completely significant ($0.02 > P > 0.01$).*

The results of the determinations of the K^{42} spaces in the heart and skeletal muscle in normal and vagus-stimulated toads after short distribution periods (< 20 minutes) are shown in figures 26 and 27. The K^{42} spaces in the atrium and ventricle in the vagus-stimulated hearts are significantly larger than in

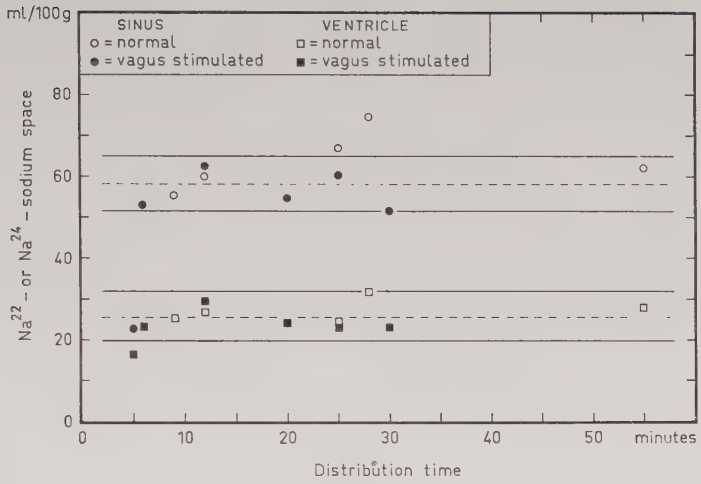

Figure 23. The Na^{22}-or Na^{24}-sodium spaces in the sinus venosus and ventricle in normal and vagus-stimulated toads after short distribution periods. The lines show the mean value $\pm$ 2 S.D. for the Na^{24}-sodium spaces after distribution periods of 22—50 hours (see chapter VI).

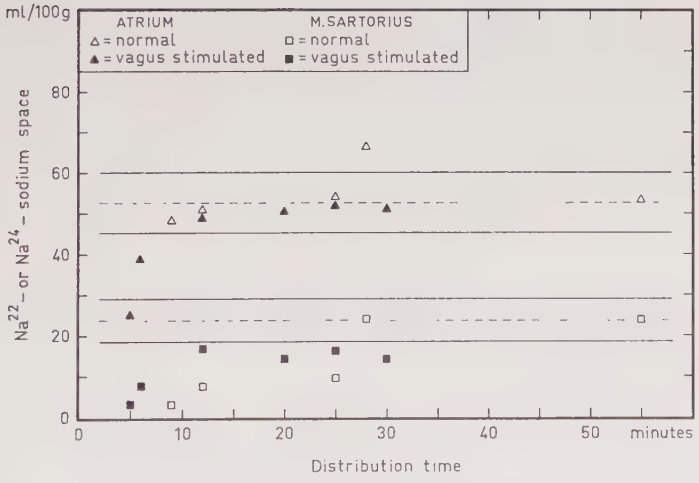

Figure 24. The Na^{22}-or Na^{24}-sodium spaces in the atrium and sartorius muscle in normal and vagus-stimulated toads after short distribution periods. The lines show the mean value $\pm$ 2 S.D. for the Na^{24}-sodium spaces after distribution periods of 22—50 hours (see chapter VI).

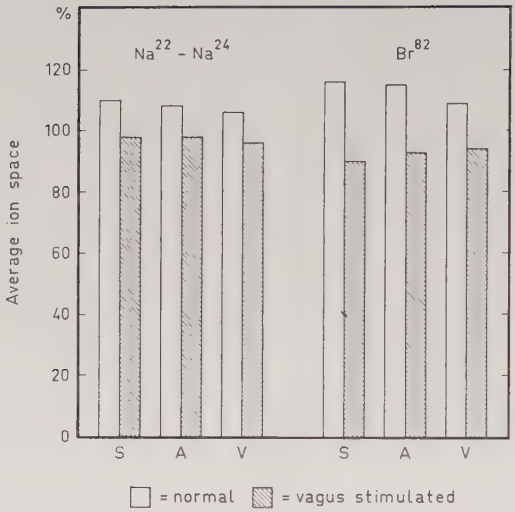

Figure 25. Mean values of Br^{82}-bromide and Na^{22}- Na^{24}-sodium spaces in normal and vagus-stimulated hearts after short distribution periods (9—30 minutes) expressed as percentages of the mean value of the corresponding Br^{82}-bromide spaces or Na^{24}-sodium spaces found with distribution periods of 22—50 hours (see Table 12, page, 60).

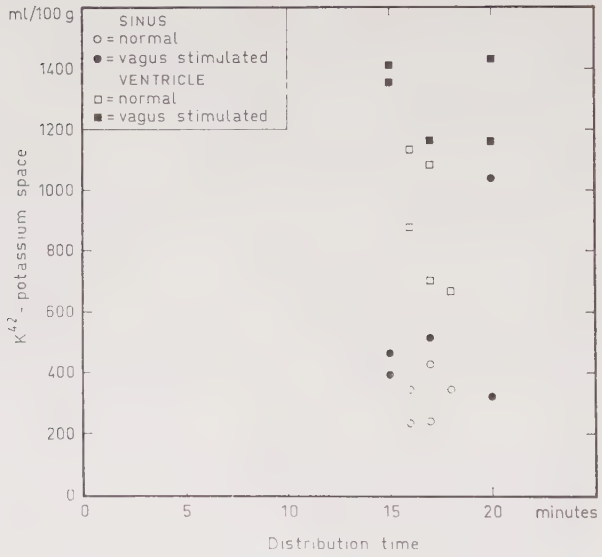

Figure 26. The K^{42}-potassium spaces in the sinus venosus and ventricle in normal and vagus-stimulated toads after short distribution periods.

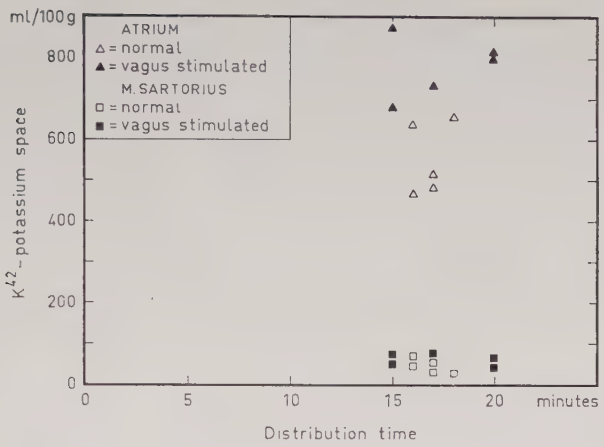

Figure 27. The K^{42}-potassium spaces in the atrium and sartorius muscle in normal and vagus-stimulated toads after short distribution periods.

the normal hearts ($P < 0.01$) while in the sinus there is no significant difference between the normal and vagus-stimulated hearts ($P > 0.10$).

No difference was observed in the K^{42} spaces in the skeletal muscle between normal and vagus-stimulated toads ($P > 0.5$). Although vagus stimulation is associated with circulatory collapse it does not seem likely that this latter would produce an observable effect as it exists for so short a time. Comparison of the K^{42} spaces in the skeletal muscle (sartorius) before and after vagal stimulation in the same toads showed no difference (these results are not given in the figure).

Relative K^{42} content in the heart after a long distribution period

Figures 28–30 show the relative K^{42} content in the heart (in relation to skeletal muscle) after a long distribution period (10–32 hours). There is no difference between normal and vagus-stimulated hearts. After this relatively long distribution period the K^{42} is probably almost completely equilibrated with stable, easily exchangeable potassium in the heart, and the relative K^{42} concentration in the heart should therefore be a

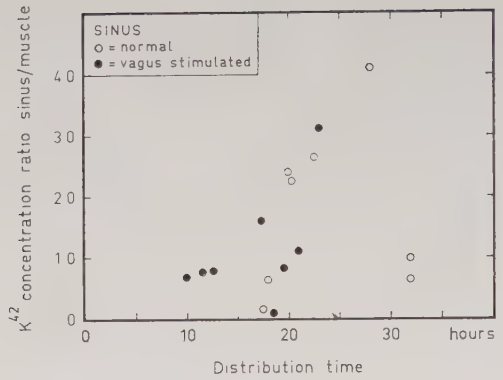

Figure 28. The relative K^{42} concentration in the sinus venosus (sinus/sartorius muscle) in normal and vagus-stimulated toads.

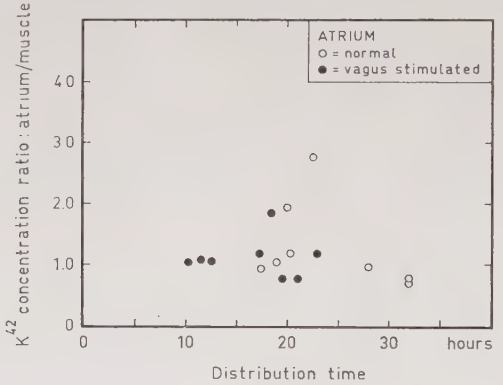

Figure 29. The relative K^{42} concentration in the atrium (atrium/sartorius muscle) in normal and vagus-stimulated toads.

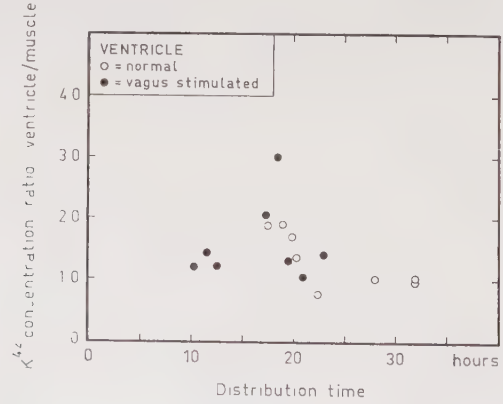

Figure 30. The relative K^{42} concentration in the ventricle (ventricle sartorius muscle) in normal and vagus-stimulated toads.

measure of the *total* potassium content, or at least of the easily exchangeable potassium if this ion is functionally compartmentalized in the cells. Since there appears to be no difference between normal and vagus-stimulated hearts, this seems to indicate that *vagal stimulation does not cause any obvious increase in the potassium concentration* (cf. page 85).

The total content of sodium and potassium

The contents of sodium and potassium in the heart and skeletal muscle tissue (sartorius) in normal and vagus-stimulated toads are shown in Table 18. The values represent the mean values. There are no differences between the normal and vagus-stimulated toads ($P > 0.2$ for all cases except with regard to skeletal muscle where $0.05 > P > 0.02$).

The S^{35}-sulphate spaces in normal and vagus-stimulated hearts

Figures 31 and 32 show the S^{35}-sulphate spaces in the heart and skeletal muscle of normal and vagus-stimulated toads after short distribution periods. There are no differences between the sulphate spaces in normal and in vagus-stimulated hearts.

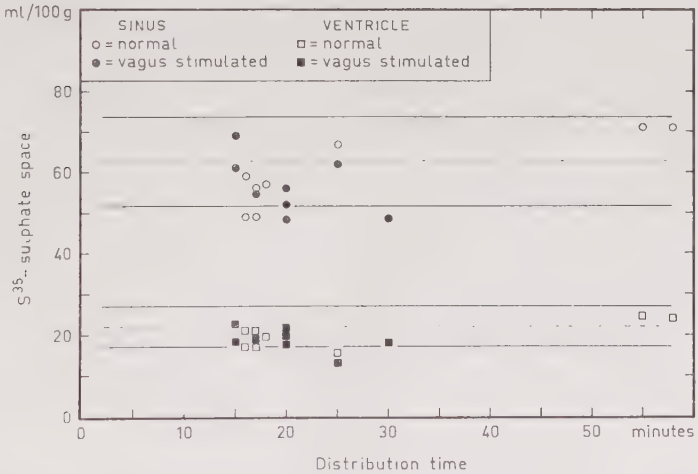

Figure 31. The S^{35}-sulphate spaces in the sinus venosus and ventricle in normal and vagus-stimulated toads after short distribution periods. (The Na^{24}-sodium and Br^{82}-bromide *or* K^{42}-potassium spaces were determined at the same time). The lines show the mean value ± 2 S.D. for the S^{35}-sulphate spaces after distribution period of 22—50 hours (see chapter VI).

Table 18.

The sodium and potassium concentrations in the cardiac and skeletal muscle in normal and vagus-stimulated toads (in captivity <3 months). The table shows the mean values, standard deviation and number of determinations.

Preparation	Sodium				Potassium			
	Normal meqv/kg wet tissue	No. of determinations	Vagus-stimulated meqv/kg wet tissue	No. of determinations	Normal meqv/kg wet tissue	No. of determinations	Vagus-stimulated meqv/kg wet tissue	No. of determinations
Sinus venosus	77.4 ± 10.3	14	72.9 ± 14.1	10	24.5 ± 2.0	14	24.4 ± 7.2	11
Atrium	62.3 ± 7.8	14	58.3 ± 9.6	11	35.7 ± 5.4	14	32.4 ± 7.7	11
Ventricle	37.9 ± 5.5	14	38.2 ± 6.0	17	59.5 ± 5.8	14	60.2 ± 8.8	17
Skeletal muscle	24.2 ± 3.5	20	23.1 ± 3.2	13	72.3 ± 3.7	20	75.3 ± 3.8	13

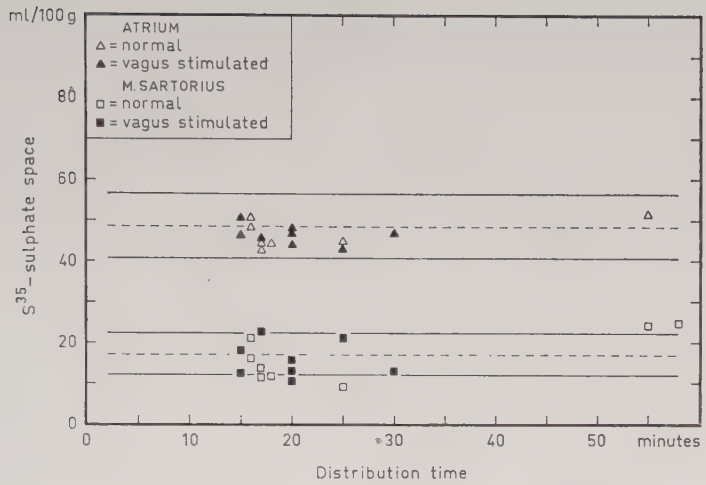

Figure 32. The S^{35}-sulphate spaces in the atrium and sartorius muscle in normal and vagus-stimulated toads after short distribution periods. (The Na^{24}-sodium and Br^{82}-bromide *or* K^{42}-potassium spaces were determined at the same time). The lines show the mean value ± 2 S.D. for the S^{35}-sulphate spaces after distribution periods of 22—50 hours (see chapter VI).

DISCUSSION

The results obtained show that vagal stimulation increases transport of K^{42}, while there appears to be no effect on the rate of influx of sulphate ions. There are several possible explanations for the increased influx of K^{42} into the heart, e. g. an increase in the concentration of potassium in the heart, i. e. a net flow of potassium from the blood into the heart or an increased exchange between the heart and the blood *without a net flow* of potassium. Since it is known (SJÖSTRAND, 1964) that *in vitro* the extracellular space in the toad sinus and atrium very rapidly reaches nearly the same specific activity as the perfusion solution (analogous to plasma), and the $S^{35}O_4$ experiments reported here support this, it seems most likely that the increased transport of K^{42} is between the extra- and intracellular spaces. This increased transport of ions was observed both in the atrium and ventricle and occurred in the absence of any observed net inflow or outflow of potassium.

The possibility that a large increase of the extracellular space caused the transport to increase can also be excluded,

since the sulphate spaces, which may be regarded as an approximate measure of the extracellular spaces, appear to remain unchanged on vagal stimulation. Furthermore the extracellular potassium constitutes a very small proportion of the total potassium, so that even a very large increase of the extracellular space could hardly explain the increased influx of K^{42}. A large increase in the ECV would probably also give rise to increased diffusion of other ions from the surroundings (from the blood plasma to the extracellular space). No such effect was observed. It seems therefore plausible that vagal stimulation causes an increased exchange of potassium between the heart and its surroundings (blood plasma), but there is no noteworthy net flux and hence no great changes in concentration in the heart itself. As the slower step is normally between the extra- and intracellular spaces, it is probably this transport which is primarily increased by vagal stimulation.

The mean values for the K^{42} spaces in the vagus-stimulated atria and ventricles are considerably greater (approximately 1.4 times) than the corresponding mean values for the non-stimulated atria and ventricles after a distribution period of 15–20 minutes. No difference in effect ($P > 0.5$) was observed between the atrium and ventricle.

Vagal stimulation appeared to have no effect on the rate of transport of K^{42} in the sinus venosus, but this may be due to the equilibration having proceeded further in the distribution time allowed. The method used is somewhat rough and the ratio of extracellular to intracellular potassium content is the most anfavourable in the sinus venosus. In other words it cannot be excluded that the same effect does also occur in this part of the heart and this seems quite likely, since there is evidence that the principal part of the vagus fibres pass to the pacemaker of the heart (Setekleiv, 1963). Harris and Hutter (1956) found an increase in both the influx and efflux of K^{42} in isolated sinus venosus from frogs treated with acetylcholine or vagal stimulation.

The Br^{82} content in the heart relative to that in the skeletal muscle does not differ between normal and vagus-stimulated toads, but if the Br^{82}-bromide space is calculated, this is smaller in vagus-stimulated than in normal hearts, as is also

possibly the case for the Na^{22}-Na^{24}-sodium space. There are several possible explanations for these results: 1) If the radioactive isotopes are not equilibrated with the stable isotopes there may be a decrease in equilibration rate caused by the vagal stimulation. Since presumably the extracellular space is completely or almost completely equilibrated after the distribution period chosen, the difference would in this case be due to a decreased rate of exchange between the extra- and intracellular spaces. Such a difference would, however, need to be rather large to be observed because the intracellular sodium and bromide contents are very small compared with the extracellular so that this explanation seems therefore not very probable, but cannot be excluded (*vide infra*). 2) Another explanation may be that both the radioactive sodium and bromide ions were equilibrated with stable isotopes and that the results are due to a net flow of sodium and bromide from the heart either caused by the vagal stimulations as such or by the cardiac arrest produced by the stimulation (e. g. a squeezing out of extracellular fluid into the blood). Such a squeezing effect can, however, probably be excluded, since the sulphate space determinations made simultaneously with the Br^{82}-bromide and the Na^{24}-sodium spaces showed no difference between normal and vagus-stimulated hearts.

Some earlier studies (Öbrink and Essex, 1953; Klein and Holland, 1958; Holland and Cervoni, 1962) indicate that the sodium ions also are effected by vagal stimulation. Öbrink and Essex (1953), for example, found that the very high sodium concentration in the sinus node region appeared to decrease during vagal stimulation. Holland and Cervoni (1962) reported that acetylcholine increased the rate of entry of Na^{24} in quiescent or stimulated, isolated rabbit atria (equilibrated for 30–40 mins in a Na^{24}-solution).

Hutter (1961) found that acetylcholine or vagal stimulation produced no effect on the bromide ion transport in the frog sinus venosus. It is not easy, however, to observe changes in the flows of sodium or bromide ions between the extra- and intracellular spaces, even if these changes are large, since the intracellular contents of these ions are small.

As mentioned the chloride ions are relatively unimportant

to the resting potential, but may possibly be of great importance to the action potential. If furthermore the cell membrane contains fixed negative charges, as suggested by TEORELL (1936), it will be doubtful whether any noteworthy flows of anions occurs during the action potential, and it is also uncertain whether vagal stimulation changes the rate of equilibration of Br^{82} in the heart. It should be mentioned, however, that it is not inconceivable that the charge of the cell membrane may become changed under the influence of acetylcholine, whereby vagal stimulation could affect the rate of transport of the bromide ions also.

The length of the distribution period

SJÖSTRAND (1964) found that a perfusion period of about 5–10 minutes for isolated sinus venosus and 10–15 minutes for isolated atrium was required for attainment of maximal activity of Na^{24} and Br^{82}. Subsequent to these periods the activity in the preparations increased only negligibly. During the shorter distribution periods used (5–15 minutes) the Br^{82} and Na^{24} would probably have hardly had time to distribute in the toads completely, at least not in the ventricle.

CONCLUSIONS

The experiments described here show that *the rate of transport of K^{42} in the heart increases on vagal stimulation* (because of changes in driving forces, the permittivity of the cell membrane or possibly other unknown factors). *The bromide and possibly also the sodium spaces (determined with radioactive isotopes) appeared to be smaller in vagus-stimulated than in normal hearts, which may be explained in several ways* (changed transport rate and/or net flow from the heart). *There was no effect* of *vagal stimulation on the sulphate spaces.*

CHAPTER X

General summary

The aim of this investigation was to study the electrolyte distribution in the heart both under normal conditions and under the influence of vagal stimulation.

In *chapter I* a short review is given of earlier studies of the electrolyte composition in biological tissues. The extra- and intracellular phases in a tissue are defined, and the procedure for the determination of the extracellular volume is described briefly. The aim of this investigation was to determine the electrolyte distribution in different regions of the heart and to compare this distribution in the heart with that in the skeletal muscle. A further aim was to study the electrolyte distribution in the vagus-stimulated heart, and the exchange of ions between the heart and the blood under the influence of vagal stimulation.

Chapter II describes the anatomy and physiology of the toad heart and also all the procedures and chemical and radioactive methods of analysis used.

Chapter III reports the results of determinations of the sodium and potassium contents in different regions of the heart and skeletal muscle (sartorius). *The concentration of sodium and potassium is not the same in any heart region or skeletal muscle* (for Na: sinus venosus $>$ atrium $>$ ventricle $>$ sartorius; for K: sinus venosus $<$ atrium $<$ ventricle $<$ sartorius). *The results indicate that if toads are kept in captivity for a long period the K content in the heart and the sartorius muscle decreases, while the Na content does not appear to change.*

In *chapter IV* data on the distribution of chloride in the heart are reported. A review is also given of earlier methods for the analysis of chloride in biological tissue and of previous investigations on the distribution of chloride in the heart and skeletal muscle. The results of the chloride determinations in different regions of the heart and in the skeletal muscle are given. *The pattern of chloride is similar to that of Na:* sinus venosus > atrium > ventricle > sartorius muscle.

In order to calculate the electrolyte distribution in the extra- and intracellular compartments the size of the extracellular space must be known. Since the chloride space is considered to be a good approximation in many tissues of the extracellular space, this was calculated in different regions of the heart and in the sartorius muscle (the mean values in ml/100 g wet tissue: for sinus 75, for atrium 50, for ventricle 25 and for sartorius 15). *The chloride spaces so calculated were also compared with the corresponding* Br^{82}*-bromide and* I^{131}*-iodide spaces (no differences were found).*

Chapter V describes studies of the distribution spaces for different carbohydrate molecules and sulphate ions, which appear to remain essentially extracellular. The results showed that *the size of the mannitol and sucrose spaces continued to increase greatly after a long distribution period, which was interpreted as indicating entry of these species into the cell.* Maltotriose was less suitable as an extracellular indicator, since the distribution spaces for this substance were extremely large, probably due to its breakdown in the tissue of the toad. *Sulphate seemed to give the best estimate of the extracellular space, at least in the heart, since the sulphate ions very rapidly equilibrated with a volume which probably did not subsequently increase even after a long distribution period* (the mean values for the sulphate spaces in ml/100 g wet tissue were: sinus 63, atrium 49, ventricle 22 and sartorius muscle 17).

In *chapter VI* results of simultaneous Na^{24}-sodium, Br^{82}-bromide and S^{35}-sulphate determinations are compared. *There were no differences between the* Na^{24}*-sodium and* Br^{82}*-bromide spaces in the atrium, ventricle and sartorius muscle, while in*

the sinus venosus the Br^{82}-bromide space was larger than the Na^{24}-sodium space. In the skeletal muscle the S^{35}-sulphate space was smaller than the Na^{24}-sodium space, but in the heart there were no significant differences between the S^{35}-sulphate spaces and the other ionic spaces determined. The simultaneous determinations made possible more accurate comparisons between different extracellular space approximations and more reliable calculations of the intracellular electrolyte concentrations.

In *chapter VII* the results of the different extracellular space determinations in the different heart regions and the skeletal muscle are discussed. *The sulphate space, which seems to be the best extracellular indicator, and other ionic spaces in all heart regions were after short distribution periods larger than the mannitol and sucrose spaces. This may be due to some hindrance in the extracellular equilibration of the latter solutes.*

The extracellular space is different in each region of the heart (sinus venosus > atrium > ventricle), regardless of which substance is used for the ECV determination.

The water content in the heart and sartorius muscle was found to constitute 80–85 % of the weight of wet tissue.

The intracellular concentrations of sodium (the estimated mean values varied between – 6 and 10 meqv/l calculated from the sulphate spaces) *and potassium* (approx. 100 meqv/l calculated from the sulphate spaces in newly captured toads) *were of the same order of size in all regions of the heart, while the intracellular chloride concentration was not the same* (mean values calculated from the S^{35}-sulphate spaces, cf. Table 15, line 1, page 67; expressed in meqv/l: for sinus 24, for atrium 14, for ventricle 5 and for sartorius muscle 3). *Thus for Na and K the differences in total contents in different regions of the heart can be explained entirely by the fact that the extracellular volumes differ, but the variations in chloride content appear to be due partly to the differences in the extracellular volume and partly to intracellular differences.*

This chapter also discusses (a) the relationship between the histological appearance and the spaces determined, (b) factors of importance in the transmembrane transport, such as the

properties of the membrane and those of the solutes, the driving forces and the length of the distribution period. Also discussed is the Donnan equilibrium between plasma and the extracellular water, and its significance in ion distribution.

Chapter VIII reviews briefly the theories which seek to explain the ionic asymmetry between the intra- and extracellular spaces in excitable tissue, the genesis of excitability and possible mechanism of the effect of acetylcholine or vagal stimulation on the heart.

Chapter IX describes experiments which showed that *the rate of transport of potassium ions in the heart increases on vagal stimulation, while the sulphate ions do not appear to be affected. The bromide and possibly also the sodium spaces (determined with radioactive isotopes) appear to be smaller in vagus-stimulated than in normal hearts, which may be due reduced to a either equilibration rate and/or a net flow of bromide or sodium from the heart* (not so large, however, that it was observed by the analysis of stable isotopes).

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